ACTIVE SITE MAPPING STUDIES OF PIGEON LIVER FATTY ACID SYNTHETASE

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by

SANCHITA MUKHERJEE

to the

DEPARTMENT OF CHEMISTRY

INDIAN INSTITUTE OF TECHNOLOGY KANPUR

JANUARY, 1996

Dedicated to my parents

CHM - 1996 - D - MUK - ACT

STATEMENT

I hereby declare that the matter incorporated in this thesis entitled **"Active Site Mapping Studies of Pigeon Liver Fatty acid Synthetase"** is the result of investigations carried out by me under the supervision of Professor S.S. Katiyar in the Department of Chemistry, Indian Institute of Technology Kanpur, Kanpur, India.

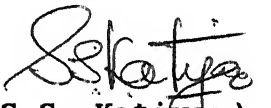
In keeping with the general practice of reporting scientific observations, due acknowledgment has been made wherever the work described is based on the findings of other investigators.

January **1996**

(**SANCHITA MUKHERJEE**)
Department of Chemistry
Indian Institute of Technology kanpur
Kanpur 208 016

CERTIFICATE I

It is certified that the work embodied in this thesis entitled "**Active Site Mapping Studies of Pigeon Liver Fatty acid Synthetase**" by Ms. Sanchita mukherjee has been carried out under my supervision and that this work has not been submitted elsewhere for a degree.



(S.S. Katiyar)
Former Professor
Department of Chemistry
Indian Institute of Technology Kanpur
Kanpur 208 016, India

and

Vice Chancellor
Shri Shahu Ji Maharaj University
Kanpur 208 024

January **1996**

DEPARTMENT OF CHEMISTRY
INDIAN INSTITUTE OF TECHNOLOGY KANPUR,
KANPUR 208 016, INDIA

CERTIFICATE II

This is to certify that Ms Sanchita Mukherjee has satisfactorily completed all the courses required for Ph.D. degree programme. These courses are:

CHM 605	Principles of Organic Chemistry
CHM 625	Principles of physical Chemistry
CHM 632	Enzyme Reaction Mechanism and Enzyme Kinetics
CHM 645	Principles of inorganic chemistry
CHM 664	Modern Physical Methods in Chemistry
CHM 681	Basic Biological Chemistry
CHM 800	General Seminar
CHM 801	Special Seminar
CHM 900	Ph.D. Thesis

Ms Sanchita Mukherjee was admitted to the candidacy of Ph.D. degree programme in March 1991 after she successfully completed the written and oral qualifying examinations.

(P. GuptaBhaya)
Convener
Departmental Postgraduate Committee
Department of Chemistry
I.I.T. Kanpur

(S. K. Dogra)
Professor and Head
Department of Chemistry
I.I.T. Kanpur

Mukunda, Anju Dixit and Manisha Nigam for their cooperation during my stay in the laboratory.

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SYNOPSIS

Fatty acid synthetase (FAS) from pigeon liver, like that from other vertebrates is a dimeric enzyme consisting of two half-molecular weight multifunctional polypeptide chains that contain the seven component enzyme activities and acyl carrier protein which in turn contains the prosthetic group phosphopantetheine required for fatty acid synthesis. These activities are acetyl-CoA and malonyl-CoA transacylases, condensing enzyme, β-ketoacyl reductase, β-hydroxyacyl dehydratase, enoyl-CoA reductase, and palmityl-thioesterase. They take part in the synthesis of long chain fatty acids from acetyl-CoA, malonyl-CoA and NADPH according to the following stoichiometric equation,

$$\text{Acetyl-CoA} + 7\ \text{Malonyl-CoA} + 14\text{NADPH} + 14\text{H}^+ \longrightarrow$$

$$\text{Palmitic acid} + 14\text{NADP}^+ + 7\text{CO}_2 + 8\text{CoA} + 6\text{H}_2\text{O}$$

It is now well recognized that FAS of all animal tissues consist of two identical subunits. Each subunit contains domains of seven different enzyme activities and the 4'-phosphopantetheine prosthetic group. However, active enzyme centrifugation studies indicated that the dimer is the only active form of this multifunctional protein because monomer

lacks β-ketoacyl synthetase activity, where two specific thiols are involved in the condensation reaction and are derived from the two subunits.

As a major outgrowth of the enzyme catalyzed reactions it has been of interest to identify the functional groups present at the active site of the enzyme for even an elementary understanding of the enzymic process. The most widely applied approaches for the identification of the active site residues are the use of chemical modification and affinity labeling methods. These techniques have been employed to characterize the active site of key enzymes of a number of metabolic pathways. This is accomplished by using reagents directed to modify distinct functional groups at the active site of the enzyme. The goal is to produce a change in the catalytic property of the enzyme that can be correlated with the functional role of specific amino acid residue.

Although extensive work has been carried on the mechanism, kinetics, enzymatic activities and properties of seven different component activities of FAS, not much information is available on the nature of amino acids at the component enzyme active sites of the FAS enzyme of pigeon liver. Hence, our objective was to identify the essential amino acid residue(s) at some of the catalytic sites of seven component activities of the pigeon liver FAS complex.

In the present study essential amino acids have been identified at the catalytic site of reductases of pigeon liver FAS enzyme. The effect of thiol specific reagents under different reaction conditions and a bifunctional fluorogenic reagent viz o-pthalaldehyde was also examined to probe the essential thiol groups present in the β-ketoacyl synthase and 4′ -phospho pantetheine prosthetic group of the pigeon liver FAS.

The thesis comprises of five chapters. Chapter I is general introduction which contains a detailed review of the relevant literature pertaining to the structure, regulation, synthesis, mechanism, purification, properties and characterization of FAS from different sources. It also includes different approaches used for the characterization of catalytic domains of the enzyme. The specific reaction of different types of chemical modifiers and how these modifiers alter the normal course of enzyme action have also been discussed.

The second chapter pertains to the isolation, purification, characterization and properties of pigeon liver FAS. The enzyme was isolated from pigeons maintained in different nutritional states. It was found that the specific activity of FAS in the liver homogenate supernatant fraction was markedly decreased when extracted from starved pigeon liver

as compared to the control. However, refeeding the birds for 48 hr resulted in significant increase in FAS activity, which was marginally higher than that observed with the control. Further, similar trend was observed with the liver weight. These results indicated that FAS activity of pigeon liver is highly dependent on nutritional status of the bird. Purification of the enzyme from the liver homogenate supernatant fraction of refed pigeon by ammonium sulfate fractionation, calcium phosphate gel treatment, DEAE cellulose column chromatography and a second ammonium sulfate treatment yielded a homogeneous preparation as ascertained by a single band in SDS polyacrylamide gel electrophoresis. Molecular weight was estimated to be 450,000. The specific activity of purified FAS was increased to 35 nmoles of palmitate formed per min per mg of protein from 2.9 U/mg in crude enzyme preparation thus resulting in 12 fold purification. This specific activity of the purified enzyme is less than the previously reported values. This may be due to the effect of environmental and some other factors on pigeons. The purified FAS showed maximum activity at high ionic strength buffer at a pH of 7.0 and 25^{o}C.

The third chapter is devoted to the studies on lysine and histidine residues present at the active site of enoyl-CoA reductase of pigeon liver FAS enzyme by the combination of reaction kinetics and chemical modification techniques. The

lysine specific reagents pyridoxal 5′-phosphate (PLP) and 2,4,6-trinitro- benzenesulphonic acid (TNBS) inactivated enoyl-CoA reductase and the overall FAS activity and the inactivation followed pseudo-first order kinetics. The reversible inhibition by PLP and spectral analysis of PLP and TNBS modified enzyme showed that the inactivation is due to specific modification of ε-amino group of lysine and not due to non-specific interactions of the inhibitor. Protection experiments showed that the modified lysine was present at or near the NADPH binding site of the enoyl-CoA reductase domain of the pigeon liver FAS. Stoichiometric studies using both the inhibitors demonstrated that two lysine residues are essential. These results suggested that one essential lysine residue is present per subunit at the enoyl-CoA reductase domain. Similarly, the FAS activity was also inactivated irreversibly by PLP in presence of light under the oxygen atmosphere and NADPH protected the enzyme against this inactivation. Photoinactivated enzyme exhibited a fluorescence maxima at 445 nm on excitation at 325 nm without reduction by sodium borohydride. The same fluorescence maxima was found in the dark reaction of FAS with PLP, only after reduction with sodium borohydride. This fact suggested light mediated addition of a nucleophilic residue (probably an imidazole group) to the pyridoxal 5′-phosphate-lysine azomethine bond. The overall FAS

and enoyl-CoA reductase activity were also inhibited by the stoichiometric concentration of diethylpyrocarbonate (DEP) which is known to react with histidine. The specificity of reaction of DEP with the histidine residue of enzyme was confirmed by the appearance of an absorption band at 240 nm and reversal of the activity of inactivated enzyme by the addition of hydroxylamine. NADPH protected the enzyme from DEP inactivation, indicating that the histidine is present at or near the NADPH binding site of the enoyl-CoA reductase. The stoichiometry of inactivation of FAS by DEP also showed that two moles of histidine residues are modified upon complete inactivation. All these results established that one essential lysine and one essential histidine residue are present per subunit at the active site of enoyl-CoA reductase of FAS. Further photosensitized PLP modification studies indicated that these lysine and histidine residues may be proximally located in the enoyl-CoA reductase domain of FAS.

In the fourth chapter we have reported the involvement of arginine residues in the catalytic functioning of pigeon liver FAS. Treatment of the enzyme by phenylglyoxal and 2,3-butanedione resulted in inactivation of overall, enoyl-CoA reductase and β-ketoacyl reductase activities of the enzyme. The rate of inactivation by 2,3-butanedione was slower than that of phenylglyoxal. All these three activities were

protected by NADPH and not by any of other substrate. The time course of inactivation of β-ketoacyl reductase and enoyl-CoA reductase activities of FAS by phenylglyoxal and 2,3-butanedione was dependent on time and concentration of the reagent and also showed almost similar inactivation pattern for both the reductase activities. The inactivation by both phenylglyoxal and 2,3-butanedione followed pseudo-first order kinetics with the second order rate constants, 0.024 $mM^{-1}min^{-1}$ and $1.2x10^{-3}$ $mM^{-1}min^{-1}$, respectively. The second order rate constants for inactivation of β-ketoacyl reductase and enoyl-CoA reductase with phenylglyoxal were nearly identical, and the sum of these values equaled to the rate constant for the inactivation of overall FAS activity. This behavior is in accordance with a mechanism whereby phenylglyoxal reacts simultaneously with residue(s), and that modification of either reductase domain results in the loss of synthetase activity. Double log plots of pseudo-first order rate constant *vs* reagent concentration yielded straight lines with slopes of unity for all three activities, suggesting, the reaction of one reagent molecule in the inactivation process. Stoichiometric results showed that four moles of arginine residues/mole of enzyme are modified upon complete inactivation. These results established the presence of an essential arginine residue at the binding site of NADPH at each of the two reductase domains of the

multifunctional FAS subunit. We have found that lysine and histidine are present in the enoyl-CoA reductase domain and further photomodification studies indicate that lysine residue is in close proximity of a necleophilic agent. In the present work we have found that arginine is also present in the enoyl-CoA reductase domain. So it is reasonable to consider that imidazole group of histidine or guanidinium group of arginine can act as nucleophilic agent in the photoinactivation of FAS.

Fifth chapter reports a detailed study on essential thiol groups of the enzyme using thiol specific reagents viz. 5,5'-dithiobis(-2 nitrobenzoic acid) [DTNB] and iodoacetamide as well as a bifunctional reagent o-phthalaldehyde. Fatty acid synthetase of pigeon liver was rapidly and reversibly inactivated by stoichiometric concentration of DTNB. The inactivation by DTNB followed a typical bimolecular process. The inactivation by DTNB was probably due to the reaction with the phosphopantetheine SH group, since it was protected by either acetyl-CoA or malonyl-CoA. Inactivation of the pigeon liver FAS by DTNB was also dependent on salt concentration. The rate of inactivation was higher in the low salt than that of high salt. Dependence of the reactivity of the SH group on salt concentration can be attributed to a salt induced conformational change affecting the local environment of this

group. The protection afforded by NADPH against the DTNB inactivation is probably due to a ligand-induced transition of FAS conformers affecting the reactivity of the susceptible SH group. We have found that further incubation of the DTNB inactivated enzyme causes cross-linking of subunits and this was examined by the electrophoretic technique. All these result suggested the proposed model for the enzyme comprising of two conformational states. Iodoacetamide also inhibited the enzyme which was protected by acetyl-CoA but not by malonyl-CoA, suggesting that this inhibitor binds at the cysteine SH site. o-phthalaldehyde is a fluorogenic bifunctional reagent and forms an isoindole derivative with a characteristic absorption maxima at 337 nm by cross linking ε-amino and sulfhydryl groups located in close proximity. The FAS was also inactivated by stoichiometric concentration of o-phthalaldehyde following a bimolecular process and partially protected by acetyl-CoA malonyl-CoA and NADPH. Fluorescence emission spectra of the o-pthalaldehyde modified FAS showed characteristic maxima at 412 nm and the fluorescence intensity was decreased in presence of acetyl-CoA and malonyl-CoA. Fluorescence spectra of the FAS enzyme preincubated with DTNB followed by o-phthalaldehyde treatment did not show the formation of isoindole derivative, whereas it was not affected when the enzyme was preincubated with iodoacetamide followed by the addition of

o-phthalaldehyde. This clearly indicates that DTNB and o-phthalaldehyde are binding to the same specific cysteine residue. Further double inhibition studies also showed that DTNB and o-phthalaldehyde, both are binding to the same specific thiol group and that was found as phosphopantetheine SH group. Similarly the FAS enzyme preincubated with TNBS followed by o-phthaladehyde treatment did not show any fluorescence indicating that both the inhibitors are binding to the same lysine residue. But enoyl-CoA reductase activity was not inhibited by o-phthalaldehyde so the lysine residues which are reacting along with the SH group in the o-phthaladehyde reaction are not essential. Quantitative analysis of o-phthalaldehyde binding with the enzyme has demonstrated that two cysteines and two lysines are involved in formation of 2 moles of isoindole derivative per mole of enzyme molecule upon complete inactivation. Similar stoichiometric result was observed with DTNB. All these results convincingly led to the conclusion that there are two phosphopantetheine-SH groups per enzyme molecule. Thus, investigations on the involvement of critical amino acid residues of reductases and condensing enzyme of pigeon liver FAS enzyme by different chemical modifiers provides a major new insight into the understanding of functional and structural similarities of subunits of the pigeon liver FAS.

CONTENTS

STATEMENT i

CERTIFICATE I ii

CERTIFICATE II iii

ACKNOWLEDGEMENTS iv

SYNOPSIS vi

CHAPTER I. GENERAL INTRODUCTION 1

I.1 Multienzyme complex 4

I.2 Biosynthesis of enzymes 5

I.3 Fatty acid synthetase 6

I.3.1 Structure of fatty acid synthetase 6

I.3.2 Regulation of fatty acid synthesis 8

I.3.3 Purification and characterisation of fatty acid synthetase 9

I.3.4 Properties of fatty acid synthetase 11

I.4 Active site of fatty acid synthetase 13

I.4.1 Active site structure 13

I.4.2 Mechanism of fatty acid synthetase 14

I.4.3. Active site mapping studies of fatty acid synthetase by chemical modification 16

I.4.3.1 Nature and behavior of essential amino acid residues 16

I.4.3.2 Group specific reagent 17

I.4.3.3 Affinity reagent 24

I.4.3.4 Selectivity 24

I.4 3.5 Reversibility 25

I.4.3.6 Stoichiometry of reaction 25

I.4.3.7 Kinetic analysis of modification ---- 26

I.4.3.7.1 Estimation of enzyme-reactant dissociation constant from the inactivation kinetics of reaction involving the formation of an intermediary complex ---- 27

I.4.3.8 Characterization of enzyme-inhibitor complex ---- 28

I.4.3.9 Location of amino acid residues in tertiary structure ---- 28

I.4.3.10 Effect of pH on enzyme inactivation ---- 30

I.4.3.11 Use of o-phthalaldehyde, a flurogenic bifunctional compound in chemical modification studies ---- 31

I.5 Objective of the present work ---- 35

References ---- 37

CHAPTER II PURIFICATION, CHRACTERISATION AND PROPERTIES OF PIGEON LIVER FATTY ACID SYNTHETASE

II.1 Introduction ---- 48

II.2 Experimental Procedure ---- 49

II.2.1 Materials ---- 49

II.2.2 Methods ---- 49

II.2.2.1 Production of fatty acid synthetase by pigeons maintained at different nutritional states ---- 49

II.2.2.2 Preparation of crude fatty acid synthetase from pigeon liver ---- 50

II.2.2.3 Purification of pigeon liver fatty acid synthetase ---- 51

II.2.2.3.1 First ammonium sulfate fractionation ---- 51

II.2.2.3.2 Calcium phosphate gel adsorption ---- 52

II.2.2.3.3 DEAE-cellulose chromatography ---- 52

II.2.2.3.4 Second ammonium sulfate fractionation ---- 53

II.2.2.4 Gel electrophoresis ---- 53

II.2.2.5 Assay of overall fatty acid synthetase ---- 54

II.2.2.6 Protein determination ---- 55

II.2.2.7 Calculation of specific activity of the enzyme— 55

II.2.2.8 Preparation of the model substrate and assay for the Enoyl-CoA reductase and β-ketoacyl reductase activity— 56

II.2.2.8.1 Preparation of substrates— 56

II.2.2.8.1.1 S-acetoacetyl-N-acetylcysteamine and S-crotonyl-N-acetylcysteamine— 56

II.2.2.8.1.1.a Synthesis of S-acetoacetyl-N-acetylcysteamine — 57

II.2.2.8.1.1.b Synthesis of S-crotonyl-N-acetylcysteamine — 58

II.2.2.9. Assay of the reductase activity— 59

II.2.2.10 Effect of ionic strength, pH and temperature on fatty acid synthetase activity— 59

II.3 Results and discussion— 60

II.3.1 Production of fatty acid synthetase by pigeons maintained in diffrent nutritional states— 60

II.3.2 Purification of pigeon liver fatty acid synthetase— 63

II.3.3 Effect of ionic strength, pH and temperature— 66

II.4 Summary— 71

References— 72

CHAPTER III IDENTIFICATION OF ESSENTIAL LYSINE AND HISTIDINE RESIDUES PRESENT AT THE ENOYL REDUCTASE DOMAIN OF PIGEON LIVER FATTY ACID SYNTHETASE USING CHEMICAL MODIFIERS

III.1 Introduction— 76

III.2 Experimental procedure— 78

III.2.1 Materials— 78

III.2.2 Methods— 78

III.2.2.1 Enzyme preparation and activity measurements — 78

III.2.2.2 Calculation of specific activity of the enzyme — 79

III.2.2.3 Modification of FAS with pyridoxal 5'-phosphate in dark and in light conditions— 79

III.2.2.4 Effect of substrates on the inactivation of FAS by PLP 80

III.2.2.5 Reduction of FAS-pyridoxal 5'-phosphate complex with sodoum borohydride 81

III.2.2.6 Reactivation of fatty acid synthetase after inactivation by pyridoxal 5'-phosphate 82

III.2.2.7 Spectral analysis of pyridoxal 5'-phosphate modified FAS in the dark and in light conditions 82

III.2.2.8 Stoichiometry of binding of pyridoxal 5'-phosphate with fatty acid synthetase 83

III.2.2.9 pH dependence studies of pyridoxal 5'-phosphate inactivation of fatty acid synthetase 84

III.2.2.10 Reaction of fatty acid synthetase with 2,4,6-trinitrobenzenesulphonic acid 85

III.2.2.11 Measurement of thiol content of 2,4,6-trinitrobenzenesulphonic acid modified enzyme 86

III.2.2.12 Stoichiometry of reaction of 2,4,6-trinitrobenzenesulphonic acid with FAS 86

III.2.2.13 Modification of FAS with diethyl pyrocarbonate 87

III.2.2.14 Reversal of diethyl pyrocarbonate modification of FAS with hydroxylamine 88

III.2.2.15 Spectral analysis of FAS modification by diethyl pyrocarbonate 89

III.2.2.16 Effect of pH on inhibition of FAS by diethyl pyrocarbonate 89

III.2.2.17 Spectrophotometric quantification of diethyl pyrocarbonate reaction with FAS 90

III.3 Results and discussion 90

III.3.1 Inactivation of FAS by pyridoxal 5'-phosphate 91

III.3.2 Kinetic analysis of the inactivation of FAS with PLP 94

III.3.3 Protection experiments 104

III.3.4 Spectral analysis of PLP modified FAS 106

III.3.5 Stoichiometry of inactivation of FAS by PLP 109

III.3.6 Photoinactivation of FAS using PLP as PLP as photosensitizer 110

III.3.7 Spectral analysis of photoinactivated FAS—— 111

III.3.8 Inactivation of FAS by 2,4,6-trinitrobenzen-sulphonic acid—— 116

III.3.9 Determination of number of lysine residues in reaction of FAS and TNBS—— 118

III.3.10 Modification of FAS by diethyl pyrocarbonate—— 121

III.3.11 The effect of substrates and substrate analogs on the inactivation by diethyl pyrocarbonate—— 121

III.3.12 Characterization of the product formed by the reaction of diethyl pyrocarbonate with FAS—— 124

III.3.13 Stoichiometry of the reaction of FAS with diethyl pyrocarbonate—— 125

III.4 Summary—— 128

References—— 130

CHAPTER IV EVIDENCE FOR ARGININE RESIDUES PRESENT AT THE β-KETOACYL REDUCTASE AND ENOYL-CoA REDUCTASE DOMAINS OF THE MULTIFUNCTIONAL FATTY ACID SYNTHETASE OF PIGEON LIVER

IV.1 Introduction—— 135

IV.2 Experimental procedure—— 136

IV.2.1 Materials—— 136

IV.2.2 Methods—— 137

IV.2.2.1 Enzyme preparation and activity measurements—— 137

IV.2.2.2 Calculation of specific activity of the enzyme—— 138

IV.2.2.3 Inactivation studies of FAS with phenylglyoxal—— 138

IV.2.2.4 Effect of substrates and substrate analogs on inactivation of FAS by phenylglyoxal—— 139

IV.2.2.5 Modification of FAS with 2,3-butanedione—— 139

IV.2.2.6 Effect of substrates and substrate analogs on inactivation of FAS by 2,3-butanedione—— 139

IV.2.2.7 pH dependence studies of inactivation of FAS by phenylglyoxal—— 140

IV.2.2.8 Stoichiometry of bindung of phenylglyoxal with FAS—— 140

IV.3 Results and discussion ---- 141

IV.3.1 Inactivation of FAS by phenylglyoxal ---- 141

IV.3.2 Effect of substrates and substrate analogs on inactivation of FAS with phenylglyoxal ---- 149

IV.3.3 Modification of FAS by 2,3-butanedione ---- 150

IV.3.4 Effect of substrates and substrate analogs on FAS inactivation by 2,3-butanedione ---- 152

IV.3.5 Effect of pH on FAS inactivation by phenylglyoxal ---- 155

IV.3.6 Number of essential arginine amino acid residue(s) modified by phenylglyoxal ---- 157

IV.4 Summary ---- 159

References ---- 160

CHAPTER V STUDIES ON THE REACTIVITY OF THE ESSENTIAL SULFHYLDRYL GROUPS OF PIGEON LIVER FATTY ACID SYNTHETASE USING THIOL SPECIFIC REAGENTS AND O-PHTHALALDEHYDE, A BIFUNCTIONAL REAGENT

V.1 Introduction ---- 162

V.2 Experimental procedure ---- 163

V.2.1 Materials ---- 163

V.2.2 Methods ---- 163

V.2.2.1 Enzyme preparation and assay ---- 163

V.2.2.2 Inactivation of FAS by thiol specific reagent DTNB ---- 164

V.2.2.3 Effect of salts on inactivation of FAS by DTNB ---- 164

V.2.2.4 Effect of substrates on inactivation of FAS by DTNB ---- 165

V.2.2.5 Reactivation of DTNB inactivated FAS by DTT ---- 166

V.2.2.6 Cross-linking studies ---- 166

V.2.2.7 SDS polyacrylamide gel electrophoresis of DTNB modified enzyme ---- 166

V.2.2.8 Stoichiometry of DTNB modified FAS ---- 167

V.2.2.9 Inactivation of FAS by iodoacetamide ---- 167

V.2.2.10 Inactivation of FAS by o-phthalaldehyde ---- 168

V.2.2.11 Effect of substrates on inactivation of FAS by o-phthalaldehyde 168

V.2.2.12 Spectral studies on o-phthalaldehyde binding with FAS 169

V.2.2.13 Stoichiometry of the reaction of FAS with o-phthalaldehyde 169

V.2.2.14 Effect of DTNB iodoacetamide and TNBS preincubation of FAS on o-phthalaldehyde binding 170

V.2.2.15 Double inhibition studies using DTNB and o-phthalaldehyde 170

V.3 Results and discussion 171

V.3.1 Kinetics of inactivation of FAS by DTNB 171

V.3.2 Effect of salts on the rate of inactivation of FAS by DTNB 173

V.3.3 Effect of substrates on the inhibition of FAS activity by DTNB 177

V.3.4 Cross-linking studies 179

V.3.5 Reactivation of DTNB inactivated FAS by treatment with DTT 182

V.3.6 Determination of number of SH groups involved in inactivation of FAS by DTNB 184

V.3.7 Effect of acyl-CoA esters on the time course inactivation of FAS by iodoacetamide 186

V.3.8 Inactivation of FAS by a bifunctional reagent o-phthalaldehyde 188

V.3.9 Effect of substrates on OPA inactivation of FAS 189

V.3.10 Characterization of isoindole formation due to fatty acid synthetase and o-phthalaldehyde reaction 192

V.3.11 Spectral analysis of OPA-modified FAS in presence of different inhibitors and substrates 194

V.3.12 Stoichiometry of the reaction of FAS with o-phthalaldehyde 197

V.3.13 Double inhibition studies 198

V.4 Summary 201

References 204

CONCLUSIONS ——— xxiv

LIST OF RESEARCH PUBLICATIONS ——— xxvii

VITAE ——— xxi

CONCLUSIONS

The studies presented in the thesis are focused to understand the nature of the active site and involvement of specific amino acid functional groups of pigeon liver FAS. Different approaches have been applied using variety of chemical modifiers to identify essential and nonessential amino acids. Analysis of the studies on structural and functional behaviour of FAS led us to draw the following conclusions:

1. Pigeon liver FAS level was highly dependent on the nutritional status of the bird. FAS level decreased significantly in the starved birds as compared to the fed birds. Refeeding of the starved bird enhanced the FAS level. FAS was purified to homogeneity and it's properties have been studied.

2. Lysine and histidine residues were identified as essential amino acids for FAS activity and were shown to be present in enoyl-CoA reductase domain. Inactivation of FAS by lysine specific reagent (PLP and TNBS) and histidine reagent diethyl pyrocarbonate was prevented by NADPH indicating that these amino acid residues are present in the coenzyme binding

site of enoyl-CoA reductase. Stoichiometric results showed that two moles of essential lysine and histidine residues are present per mole of enzyme.

3. Spectral studies of photomodified FAS indicated that lysine in enoyl-CoA reductase domain is in close proximity of a nucleophilic agent which can be either histidine or arginine which have been shown essential for FAS.

4. FAS was inactivated by arginine specific modifying reagent phenylglyoxal and 2,3-butanedione. Arginine has found to be essential for both β-ketoacyl reductase and enoyl-CoA reductase activity and was located at NADPH binding site. The rate constant of inactivation of these reductases were similar and summation of these rate constants yielded the rate for overall FAS. Stoichiometric analysis revealed that four arginine residues are crucial for catalysis.

5. Thiol specific inhibitor DTNB and iodoacetamide inhibited the FAS. Electrophoresis studies indicated that DTNB inactivation leads to the cross linking of FAS giving higher molecular weight derivative. Protection studies indicated that DTNB specifically modified pantetheine -SH group, wheras iodoacetamide reacted with cysteine -SH group.

6. A bifunctional reagent, o-phthalaldehyde inhibited FAS. Fluorescence studies have established that reaction of o-phthalaldehyde with -SH and ε-amino groups results in the

characteristic cyclic isoindole derivative formation.

7. Spectral analysis and double inhibition studies revealed that DTNB and o-phthalaldehyde reacts with pantetheine -SH group. $-NH_2$ group of FAS in o-phthalaldehyde reaction was found to be nonessential as o-phthalaldehyde did not inactivated the enoyl-CoA reductase activity which was found to contain an essential lysine residue.

In the present investigations, two pantetheine -SH groups, two lysine, two histidine and four arginine residues per mole of FAS were found to be essential for the FAS activity. Lysine and histidine residues are located specifically at the enoyl-CoA reductase domain, whereas arginine residues are present in both enoyl-CoA reductase and β-ketoacyl reductase domain of FAS.

These results lead to the conclusion that pigeon liver FAS, a multifunctional dimeric enzyme, contains two identical subunits like other animal FAS. Each subunit of FAS contains one enoyl-CoA reductase domain having one lysine one histidine residue located at the NADPH binding site. Further, stoichiometry for the arginine residues suggested that each subunit of FAS contains one enoyl-CoA reductase and one β-ketoacyl reductase domain having one essential arginine residue located at each of these active sites. Previous findings have also shown that each peptide contains a covalently attached phosphopantetheine and a thioesterase domain. All these results support the homodimer model of pigeon liver FAS.

LIST OF RESEARCH PUBLICATIONS

1. *Inhibition of pigeon liver fatty acid synthetase by specific modification of lysine residues with 2,4,6-trinitrobenzene sulphonic acid.*

Sanchita Mukherjee and S.S Katiyar Biochemistry and Molecular Biology International **1995** (in press).

2. *Evidence for the essential histidine at the NADPH binding site of enoyl-CoA reductase domain of pigeon liver fatty acid synthetase.*

Sanchita Mukherjee and S.S. Katiyar Journal of Enzyme Inhibition **1995** (in press).

3. *Inactivation of enoyl-CoA reductase in pigeon liver fatty acid synthetase by PLP: Evidence for the presence of one lysine residue at the active site.*

Sanchita Mukherjee and S.S. Katiyar Journal of Enzyme Inhibition **1995** (submitted for publication).

4. *Modification of essential lysine in pigeon liver fatty acid synthetase by pyridoxal 5'-phosphate-sensitized photooxidation: Evidence for the presence of a nucleophilic residue in close proximity of lysine residue.*

Sanchita mukherjee and S.S Katiyar Biochemical. Biophysical. Research. Communication, **1995** (submitted for publication).

5. *Investigation of the nature of o-phthalaldehyde reaction with pigeon liver fatty acid synthetase.*
Sanchita Mukherjee and S.S. Katiyar Biochimica Biophysica Acta **1996** (submitted for publication).

6. *Effect of arginine modifying reagents on pigeon liver fatty acid synthetase: Evidence for the presence of essential arginine residues at the β-ketoacyl reductase and enoyl-CoA reductase domain.*
Sanchita Mukherjee and S.S. Katiyar Biochemistry and Molecular Biology International (To be submitted for publication).

7. *The effect of 5,5'-dithiobis-(2-nitrobenzoic acid) on the reactivity of the essential sulfhydryl groups present in the pigeon liver FAS.*
Sanchita Mukherjee and S.S. Katiyar Biochemistry and Molecular Biology International (to be submitted for publication).

VITAE

The author was born on June 25, 1966 in **Calcutta**, India. She passed the Secondary and Senior Secondary School Examination conducted by **"West Bengal Board of secondary Education"**, Calcutta in 1980 and 1982, respectively. She completed B.Sc. (Hons.) **Chemistry** from Bethune College, **"Calcutta University"**, Calcutta, in 1986. She did her Masters in **Biochemistry** from **"Calcutta University"**, Ballygunge Science College calcutta in 1988.

She joined the Ph.D. program in December 1989 in the Department of Chemistry, **"Indian Institute of Technology Kanpur, Kanpur** where she received Junior and Senior Research Fellowships through NET examination conducted by Joint UGC and CSIR. Presently, she is continuing as Senior Research Fellow in the same department.

CHAPTER I

GENERAL INTRODUCTION

The synthesis of long chain fatty acids from acetyl-CoA and malonyl-CoA involves several sequential reactions and acyl intermediates. The fatty acid synthesising enzyme systems are present as soluble entities in the cytoplasm of plant, animal and microbial cells. Fatty acid synthesizing multienzyme complexes can be classified into two different types: i) Nonaggregated and ii) aggregated. Nonaggregated form do not have a higher level of structural organisation. They occur in many bacteria [1-3] and plants [4-6] and their component enzymes exist as individual proteins in ruptured cell extracts. Their enzymes are separable and several have been partially or completely purified by standard methods of purification [7,8]. The E. coli fatty acid synthesizing system consists of seven individual enzymes and a pantetheine containing protein called acyl carrier protein (ACP) which were separated in active form

and studied individually [9]. Irrespective of the source, size and the architecture, whether aggregated or nonaggregated enzyme system, the synthesis of fatty acids involves the same chemical mechanism and stoichiometry through a series of highly organised chemical reactions. The nature of these reactions and intermediates involved in fatty acid synthesis is known primarily from studies of fatty acid synthesis in cell-free extracts of E.coli [9,10]. Aggregated fatty acid synthesizing multienzyme complex occurs in avian liver [11] and specialised glands [12,13] mammalian liver and mammary gland [14-19] and microbial organism like yeast [20] and Mycobacterium smegmatis [21].

The yeast FAS is a complex of two nonidentical subunits, α and β and exists as $\alpha_6\beta_6$ structure. Stoops and Wakil [22] have reported the separation of the α and β subunits each containing the catalytic centers for some of the partial reactions required for the fatty acid synthesis. Whereas animal FAS have two identical subunits and each polypeptide contains the catalytic sites for the seven partial reactions required for palmitate synthesis. It is known that multifunctional proteins are actually arranged as a series of globular domains which are the sites of catalytic or regulatory activity, that are connected by polypeptide bridges sensitive to proteolytic attack and such domains are readily discernible in the electron

micrograph of the rat liver FAS [23,24]. More recently, chicken liver FAS was proteolysed by a variety of proteases, employed individually or in combination. The proteolytic profiles were analysed with respect to both kinetics and the size of the peptide fragments and the data suggested that the polypeptide subunits of chicken liver FAS are identical [25]. The differences in the eukaryotic and prokaryotic enzyme system, vis a vis the structural organisation, the mechanism of some of the reactions involved in yeast FAS, the presence of eight distinct functions on the single polypeptide chain of the animal FAS, have stimulated investigations with respect to relative location, functional organisation of various activities and understanding of the active site structure.

The role of enzymes as biocatalysts for reactions occurring in the living organism as well as their application in biotechnology has stimulated the investigations towards the understanding of their structural organization and mechanism of action. The characteristic feature of enzymes is their ability to catalyze a reaction at a very high rate under mild conditions. Several approaches have been used to understand the enzyme mechanism. They are reaction kinetics (26,27), chemical modification (28-29), site directed mutagenesis (30,31), X-ray diffraction (32,33), nuclear magnetic resonance (34,35) and monoclonal antibodies (36,37). By studying the reaction

kinetics one can learn about the order in which substrates bind and products are released from the enzyme. However, information regarding the molecular details of mechanism by which these transformations take place and how the structural elements contribute to the process are not obtained. By the combination of reaction kinetics with chemical modification technique a great deal of information regarding the structural and functional aspects of the enzyme can be obtained.

I.1 MULTIENZYME COMPLEX

Several enzyme activities catalysing different consecutive reactions are located at different sites of the same macromolecular complex, which cannot be fractionated into smaller molecules, bearing the individual enzyme activities. Such a nondissociable combination of enzyme activities in the same macromolecule, constitues a multienzyme system. This class of enzyme is involved in the biosynthesis of fatty acids. Animal fatty acid synthetases are the known multifunctional enzymes, consisting of subunits having catalytic domains for fatty acid synthesis. These enzymes have two characteristic properties: structurally they consist of large polypeptide chains and functionally they have multiple catalytic activities on these polypeptide subunits. This implies that the active centers of the protein are generated by the folding of contiguous stretches of the polypeptide chain to yield

autonomous globular structures or domains each having a specific but different catalytic activity.

I.2 BIOSYNTHESIS OF FATTY ACIDS

Biosynthesis of fatty acid occurs predominantly in the cytosolic part of cells in liver, adipose tissues and mammary glands of higher animals. CO_2 is essential for fatty acid synthesis in the cell and it comes from HCO_3^-. The biosynthesis of long chain fatty acids occurs in two distinct steps. The first step is, the conversion of acetyl-CoA to malonyl-CoA by a reaction catalysed by a biotin containing multienzyme system named acetyl-CoA carboxylase. The second step is the conversion of acetyl-CoA and malonyl-CoA to palmitate in presence of NADPH, a reaction catalysed by fatty acid synthetase. Fatty acid synthesis remains under allosteric or short term control, primarily through the acetyl-CoA carboxylase. The allosteric regulation of carboxylase by citrate and by fatty acyl-CoA is well established [38,39]. Citrate or isocitrate activates the carboxylase by polymerisation of the inactive protomer to an active polymer. Phosphorylation of the enzyme both with cAMP-dependent and cAMP-independent kinases has been reported [40,41]. This development makes it possible to relate fatty acid synthesis to carbohydrate metabolism and to understand the roles of glucagon, catecholamines and insulin in these processes.

I.3 FATTY ACID SYNTHETASE

I.3.1 *Structure of fatty acid synthetase*

Fatty acid synthesizing multienzyme complexes can be classified in aggregated and nonaggregated form on the basis of their structure. Nonaggregated forms do not have a higher level of structural organisation. They occur in many bacteria and plants. Aggregated forms of FAS occur in avian liver and other specilised glands, mammalian liver and mammary gland and microbial organisms like yeast and Mycobacterium smegmatis. The FAS of eukaryotic cells (excluding plant cells) are complexes of multifunctional proteins and can be divided into two groups, exemplified by the synthetases isolated from animal tissues and yeasts. The animal synthetases are multifunctional proteins of molecular weight ~ 500,000 consisting of two identical subunits. They have therefore α_2 structures. The yeast synthetase is a complex of two nonidentical subunits, α(Mol. wt. 213,000) and β(Mol. wt. 203,000), with an apparent mol.wt. of 2.4×10^6, with $\alpha_6\beta_6$structure. Schweizer's genetic studies have supported also that the yeast FAS consists of two nonidentical multifunctional proteins as it is encoded by two unlinked genes [42,43]. The FAS of rat liver, adipose tissue [44] and lactating mammary gland [45], chicken liver [46,47], rabbit mammary gland [48] and uropygial gland [13] have many common features. Besides having the same size, charge and shape

they have two identical subunits. Recently it was confirmed by the finding of one mole of 4'-phosphopantetheine per subunit to the preparation of chicken [46], pigeon [49], rat [44] uropygial gland of goose [13] and of the higher bacterium, although these results contrast with the value of one prosthetic group per mole of enzyme reported earlier for the synthetases of pigeon [50] rat [51] and dog liver [18] and rat lactating mammary gland [52]. Furthermore electron microscopic studies [44] and recent genetic studies [53-55] indicate that the animal FAS is a multifunctional enzyme and subunits are identical and that each polypeptide chain contains the sites of partial reactions required for palmitate synthesis. Dissociation of the native enzyme to monomers results in the retention of all the six enzymatic activities, viz acetyl and malonyl transacylase, β-ketoacyl reductase, enoyl-CoA reductase, dehydratase and thioesterase [44,56-58]. one of activities that remains absent from the monomer is β-ketoacyl synthetase (condensing enzyme), whose active center is dependent on the presence of two juxtaposed thiols [59], each derived from one subunit. Proteolytic digestion of chiken liver FAS by various proteases has demonstrated clearly the structural organisation of FAS and further proved that FAS contains two identical subunits [25]. Animal FAS has three domains, domain-I contains the acetyl and malonyl-transacylases

as well as condensing enzyme site, thus making this domain for substrate binding and chain elongation domain. On the other hand, domain-II contains the β-ketoacyl reductase, the dehydratase and the enoyl reductase activities. The ACP and its 4'-phosphopantetheine arm is located next to the reduction domain (domain-II) connecting it to the chain termination or palmitate releasing domain (thioesterase), domain-III [25].

I.3.2 *Regulation of fatty acid synthesis*

The activity of FAS is controlled by two ways (1) Short-term or acute control which involves allosteric or metabolic regulation and (2) Long-term control involving changes in the amount of the enzymes brought about by the change in the rates of synthesis and degradation. Lipogenic enzymes of mammalian liver are known to be markedly affected by nutritional and hormonal status. The amount of purified fatty acid synthetase isolated from pigeon liver was dependent on the nutritional status of the bird. The activity of the soluble FAS is reduced during starvation. The activity of FAS is recovered by refeeding and, in some instances they are elevated beyond normal levels by feeding fat-free diets. It was shown that there is a marked difference in the response of microsomal and soluble enzymes derived from the livers of fed, starved and refed pigeons [60]. The elongation of acyl-CoA moieties by the

microsomal enzyme was not impaired in starvation. Analysis of the fatty acids formed showed that the long-chain acyl desaturase activity was eliminated on 72 hrs of starvation. The increase in synthetase activity on refeeding or insulin administration has been directly related to increaseed amounts of synthetase as determined by immunotitration of the enzyme with antisynthetase antibodies [9,61]. The synthesis of FAS is also regulated during neonatal development and differentiation [9,61]. The differentiation dependent increase in the activities of acetyl-CoA carboxylase and FAS results from an increase in the amount of both enzymes caused by enhanced rate of synthesis [62-65]. Kasturi and Joshi [66] have demonstrated that cell differentiation and induction of FAS activity are both regulated by insulin which indicates that insulin supports phenotypic expression as well as the induction of lipogenesis and FAS activity. Further studies on avian hepatocytes culture showed that both insulin and triiodothyronine were required for maximal induction of FAS [67]. Recently, Joshi & Kasturi [68] observed that antimicrotubular agents such as colchicine and vinblastin inhibit insulin induction of FAS and stearoyl-CoA desaturase.

I.3.3 *Purification and characterization of fatty acid synthetase*

Different combination and sequence of techniques like

ammonium sulfate fractionation, DEAE-cellulose chromatography, sephadex G-100 chromatography and Calcium phosphate gel adsorption have been applied for the purification of FAS enzyme. Pigeon liver FAS was purified by Bressler and Wakil [69] to an activity of 300 nmole of NADPH oxidised per min per mg of protein. Hsu et.al [11] reported the enzyme specific activity of 1000 nmoles of NADPH oxidised per min per mg of protein. Using same sequence of tecniques Muesing and Porter [70] reported specific activity of 1120-1400 nmoles of NADPH oxidised per min per mg of protein. The fatty acid synthetase enzyme systems of pigeon, chicken, rat liver and rat lactating mammary gland exist in the soluble portion of cell as the multienzyme complex with molecular weights of approximately, $5x10^5$g/mole [70]. In contrast to E.coli fatty acid synthetase, the fatty acid synthetase from yeast and other eukaryotes has large molecular weight. FAS from these sources was isolated and purified to homogeniety and confirmed by SDS gel electrophoresis [11,12-14,15]. The yeast enzyme (moleculer weight, $2.4x10^6$) appears to be composed of three functional synthetase units [71] wheras, the pigeon liver enzyme is presumably a single functional unit. The pigeon liver FAS does not contain flavin or other groups that absorb visible light, but yeast FAS contains flavin moiety. The pigeon liver enzyme also has an excess of acidic amino acid residues and contains

no heme groups, nucleic acids or nucleotides. It contains 64 to 66 sulfhydryl groups per mole which include sulfhydryls derived from 4'-phosphopantetheine moiety of the synthetase complex [72,73]. The yeast, animal and E.coli synthetases have been shown to contain 3, 2 & 1 moles of 4'-phosphopantetheine, respectively [46,74,75]. All evidences available till date suggests that 4'-phosphopantetheine acts as a prosthetic group in all fatty acid synthesizing systems. The presence of an active acyl carrier protein in the multienzyme fatty acid synthetase complexes found in yeast and animal cells was postulated based solely on the presence of 4'-phosphopantetheine in these complexes [76]. In all systems the 4'-phosphopantetheine is bound through a phosphodiester bond between the phosphate moiety and a serine residue [76], which is alkali labile. Four NADPH binding sites were found in animal FAS system by the flourescence enhancement techniques [77]. All the seven component activities were found in each of the subunit of animal FAS but in yeast FAS three activities was found in one subunit and four activity in the other subunit.

I.3.4 *Properties of fatty acid synthetase*

The properties of FAS have been extensively studied [70]. The enzyme exhibits maximum activity at an optimum temperature of 25^0C and pH of 6.7. The enzyme gets inactivated and dissociated rapidly at 0^oC. Loss of enzyme activity is

accompanied by precipitation of protein . The heat of activation for denaturation was found to be 1.6 x 10^5 calories per mole for the pigeon liver FAS. The protein has an absorption maximum at 279 nm and has no peak at 260 nm, indicating the absence of nucleotides and nucleic acids. The purified FAS is highly specific for NADPH. The substitution of NADH (0.3 mM) as the hydrogen donor in the standard assay for fatty acid synthesis resulted in the incorporation of only one seventh to one eigths of acetyl-CoA into fatty acids as compared to NADPH as the hydrogen donor [11]. Palmitoyl-CoA inhibited almost completely the reaction at concentration of 20 μM. The purified enzyme synthesises very small amounts of CoA ester of β-hydroxy β-methyl glutaric acid and 3,5-diketohexanoic acid in the absence of NADPH. Negligible amounts of mevalonic acids are formed in the presence of NADPH. The enzyme rapidly loses activity on standing in low ionic strength buffer solution, even in the presence of dithiothreitol as it dissociates into half molecular weight subunits or on exposure to heavy metal ions in the absence of EDTA. The DTT protected (reduced) subunits are rapidly reassociated and reactivated to form the active enzyme complex, not only by an increase in salt concentration but also by micromolar concentration of NADPH [78]. Reactivation with 0.2 M KCl is optimal between pH 7.3 and 8.5.

I.4 ACTIVE SITE OF FATTY ACID SYNTHETASE

I.4.1 *Active site structure*

The active site of an enzyme is the region having a particular arrangement of functional groups that are involved in catalysing the specific reaction. The primary structure of enzyme folds in such a way so as to create a region that has correct molecular dimensions, appropriate topology and optimal alignment of functional groups and hydrophobic regions to accommodate the specific substrate. To decipher the catalytic mechanism of an enzyme, it is essential to know the structural elements (functional groups of amino acids) present and the three dimensional conformation of the active site. The FAS of animal tissue is a homodimer. The subunit contains the acyl carrier protein (ACP) site, with its 4'-phosphopantetheine group, and the seven catalytic activities are tightly bound in the complex whereas they are loosely associated with each other in prokaryotic and plant cells and can readily be separated by conventional procedures. All of the component activities can be differentiated and assayed independently using appropriate substrates [22,79]. A tentative peptide map for the location and functional organisation of these activities on the synthetase subunit has been constructed. Though each subunit contains all the catalytic sites required for palmitate synthesis, but dimer is the only active form of the

multifunctional protein as the monomer lacks β-ketoacyl synthetase (condensing enzyme) activity.

I.4.2 *Mechanism of fatty acid synthetase*

The fatty acid synthetase of pigeon liver catalyses the conversion of acetyl and malonyl-CoA to a long chain saturated fatty acid in the presence of reduced nicotinamide adenine dinucleotide phosphate. The major product of the reaction is palmitic acid, but small amounts of other long chain fatty acids are also formed. The overall reaction for synthesis of palmitic acid is:

$$\text{Acetyl-CoA} + 7\ \text{Malonyl-CoA} + 14\ \text{NADPH} + 14H^{+} \longrightarrow$$

$$\text{Palmitic acid} + 14\ \text{NADP}^{+} + 7CO_2 + 8\text{CoA} + 6H_2O$$

Acetyl-CoA serves as a primer for the reaction and it is incorporates into the methyl end of the palmitic acid [80]. The synthesis of palmitic acid is acheived through the formation of a series of intermediates covalently bound to 4'-phosphopantetheine sulfhydryl group of acyl carrier protein of FAS by thioester bond. The following are enzymes and reactions involved in the synthesis of palmitate.

Acetyl transacylase

$$CH_3COS\text{-}CoA + ACP\text{-}SH \longrightarrow CH_3COS\text{-}ACP + CoA\text{-}SH$$

Malonyl transacylase

$$\begin{array}{l} COOH \\ | \\ CH_2COS\text{-}CoA \end{array} + ACP\text{-}SH \longrightarrow \begin{array}{l} COOH \\ | \\ CH_2COS\text{-}ACP \end{array} + CoA\text{-}SH$$

β-Ketoacyl-ACP synthetase(condensing enzyme)

$$CH_3COS\text{-}ACP + Enz\text{-}SH \longrightarrow CH_3COS\text{-}Enz + ACP\text{-}SH$$

$$CH_3COS\text{-}Enz + \begin{array}{l} COOH \\ | \\ CH_2COS\text{-}ACP \end{array} \longrightarrow CH_3CO\ CH_2COS\text{-}ACP + CO_2 + Enz\text{-}SH$$

β-Ketoacyl-ACP reductase

$$CH_3CO\ CH_2COS\text{-}ACP + NADPH + H^+ \longrightarrow D\text{-}CH_3CHOH\ CH_2COS\text{-}ACP + NADP^+$$

β-Hydroxyacyl-ACP dehydratase

$$CH_3CHOH\ CH_2COS\text{-}ACP \longrightarrow trans\text{-}CH_3CH{=}CH\ COS\text{-}ACP + H_2O$$

Enoyl-ACP reductase

$$CH_3CH{=}CH\ COS\text{-}ACP + NADPH + H^+ \longrightarrow CH_3CH_2CH_2COS\text{-}ACP + NADP^+$$

Thioesterase

$$CH_3(CH_2)_{14}\ COS\text{-}ACP + H_2O \longrightarrow CH_3(CH_2)_{14}COOH + ACP\text{-}SH$$

I.4.3 *Active site mapping studies of FAS by chemical modification*

Enzymes as biocatalysts catalysing the biochemical reactions in the living organisms as well as their use in biotechnology, has stimulated the investigations for the understanding of their structural organisation and mechanism of action. The chemical modification is one of the most versatile techniques for identification of functional groups of proteins and enzymes. Modification of enzyme involves binding of a chemical moiety to the side chains of amino acid residues in the enzyme which induces some measurable change in property of the enzyme. Of the 20 natural amino acids only those possessing a polar side chain are normally the targets for chemical modification. A specific chemical modification results in the quantitative binding of the functional group belonging to a unique amino acid residue without affecting other functional groups or the conformation of enzyme molecule.

I.4.3.1 *Nature and behaviour of essential amino acid residues*

The functional side chains of amino acids located at or near the active site are involved in the substrate binding and/or catalysis. These amino acid residues are thus crucial for the enzyme activity and modification of their functional

groups leads to the loss of catalytic activity. The amino acid residues possessing functional side chains which participate in the enzyme catalysis are called essential amino acid residues. Usually, the amino acids containing reactive side chains or functional groups are located at the active site of enzymes. These include acidic amino acids [glutamate and aspartate], basic amino acids [lysine, arginine and histidine], polar uncharged amino acids [serine, cysteine and tyrosine] and side chains of methionine [possessing a nucleophilic sulfur] and tryptophan [having heterocyclic indole side chain] (81). These residues are present in a specific arrangement so that the substrate molecules are able to interact and form enzyme-substrate complex. Several factors are responsible for different behaviour of active site residues. Some of the important factors are: polarity; which affects the dissociable side chains, hydrogen bonding effects; which may stabilize the neutral or ionic species, electrostatic effects (presence of charges in the vicinity of the group) and the steric effects by the other side chains (82). All these factors differentiate the essential amino acid residues from non essential ones.

I.4.3 2 *Group specific reagents*

These modifiers belong to a class which bind covalently with amino acid side chains. Many reagents have been developed

for the modification of different amino acid side chains (83). The specific binding of these chemical modifiers results either from enhanced reactivity of residues due to their local environment in the native protein or due to the specificity of the particular reagent to a site in the protein or perhaps from combination of both the effects. The reagents and reaction conditions for the modification of protein are chosen depending upon properties of the protein and the purpose of investigation. The essential residues involved in the catalysis can be selectively modified by a variety of reagents. Some of the modifiers have broader specificity *e.g.* alkylating reagents like iodoacetate or iodoacetamide react with cysteine, histidine, methionine and lysine (Fig I. 1a). Diethyl pyrocarbonate reacts readily with histidine (Fig I. 1b) but, can also react with lysine, tyrosine or cysteine residues. The reagents which react comparatively more selectively are tetranitromethane [tyrosine specific] (Fig I. 1c), phenylglyoxal (Fig I. 1d) and 2,3-butanedione [arginine specific] (Fig I. 1e) and dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium salt [tryptophan specific] (Fig I. 1f). Few other reagents such as p-chloromercuricphenyl sulfonic acid [cysteine specific] (Fig I. 1g), 5, 5'-dithiobis-(2-nitrobenzoic) acid [cysteine specific] (Fig I. 1h), pyridoxal 5'-phosphate and 2,4,6-trinitrobenzenesulphonic acid [lysine specific]

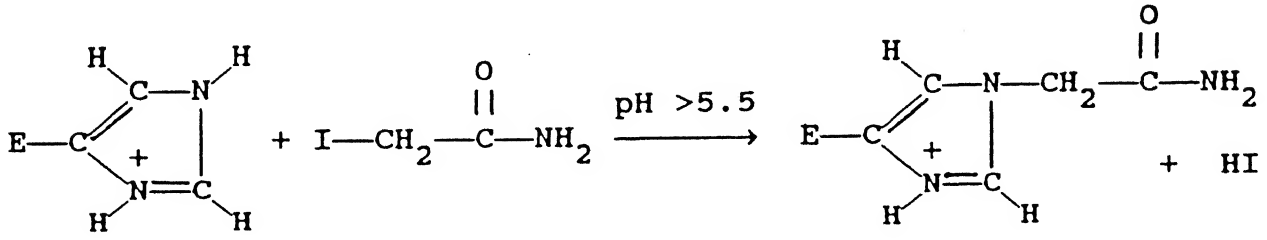
(Fig I. 1i) and (Fig I. 1j) are highly selective in nature. These chemical modifiers have been used to study the role of the essential residues in the enzyme catalysis.

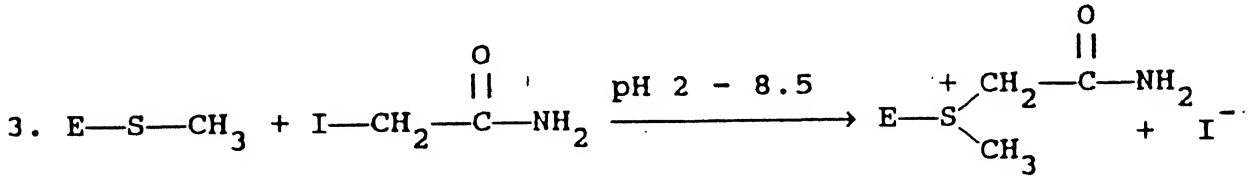
Figure I.1

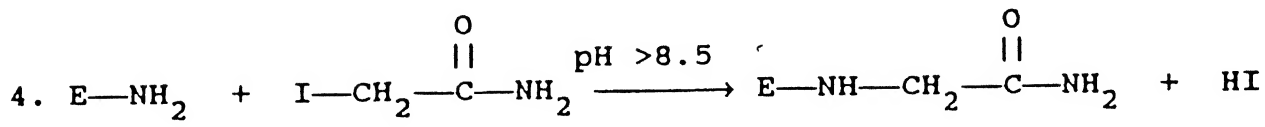
a. Modification of cysteine, histidine, methionine and lysine by Iodoacetamide.

1. $E{-}SH + I{-}CH_2{-}\overset{\overset{O}{\|}}{C}{-}NH_2 \xrightarrow{pH\ >7} E{-}S{-}CH_2{-}\overset{\overset{O}{\|}}{C}{-}NH_2$

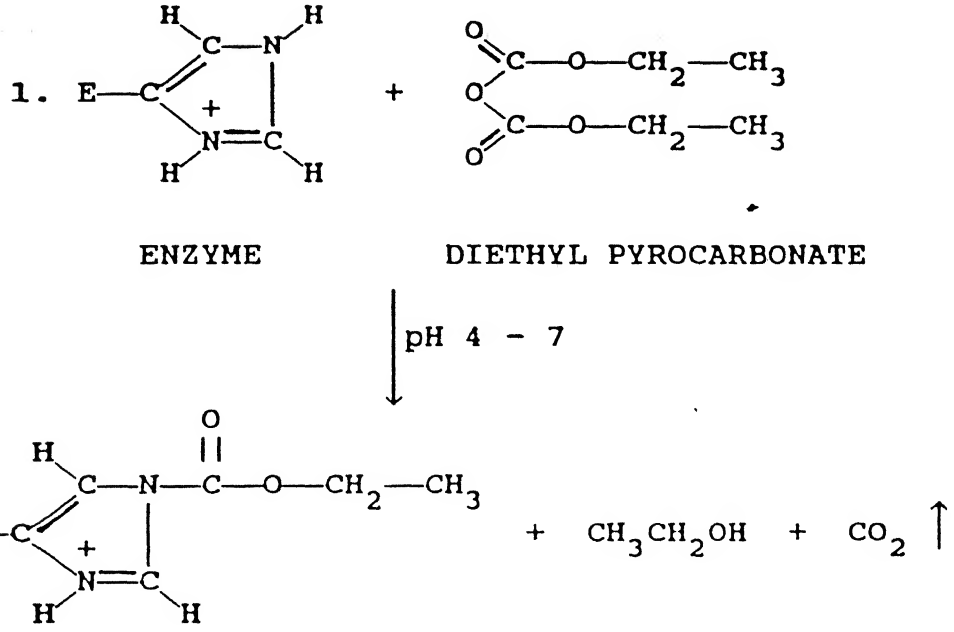
   ENZYME IODOACETAMIDE

2. 

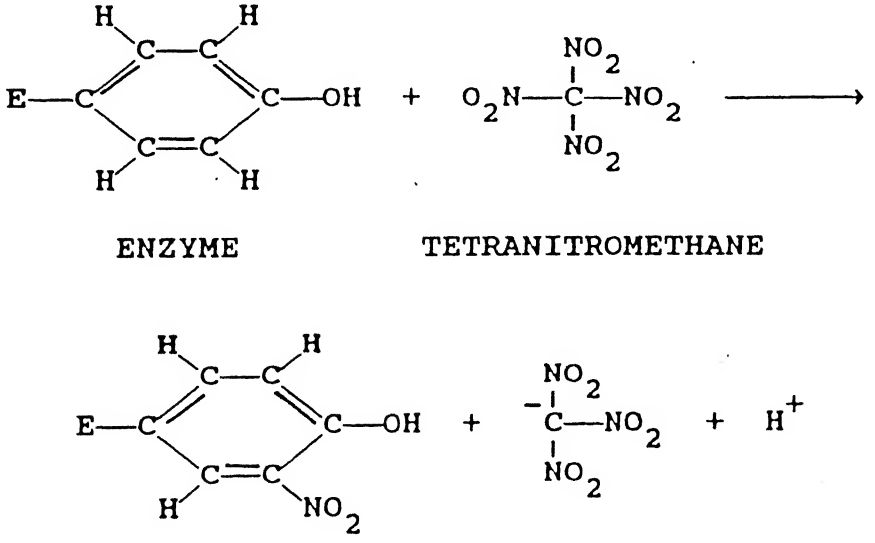
3. 

4. 

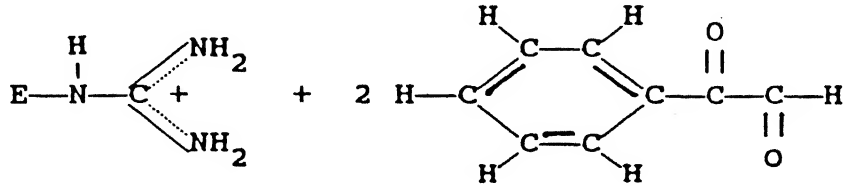
. Modification of histidine by Diethyl pyrocarbonate



:. Modification of tyrosine by tetranitromethane

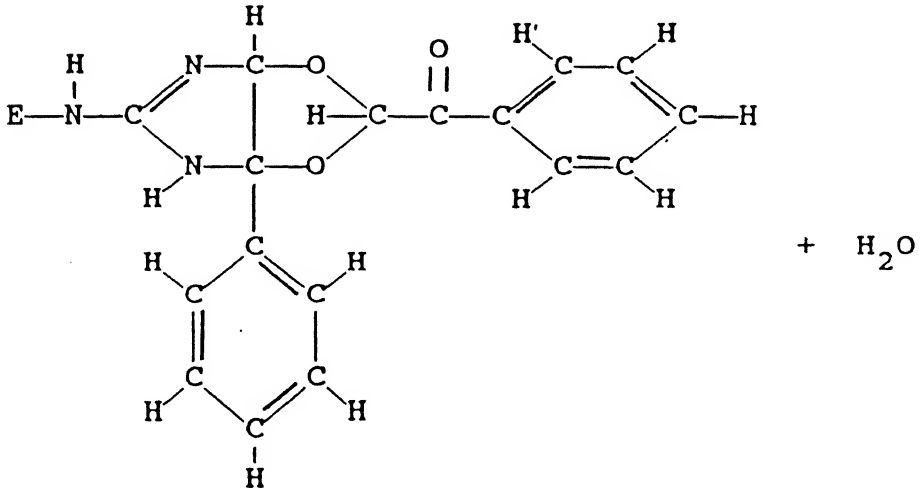


d. Modification of arginine by phenylglyoxal



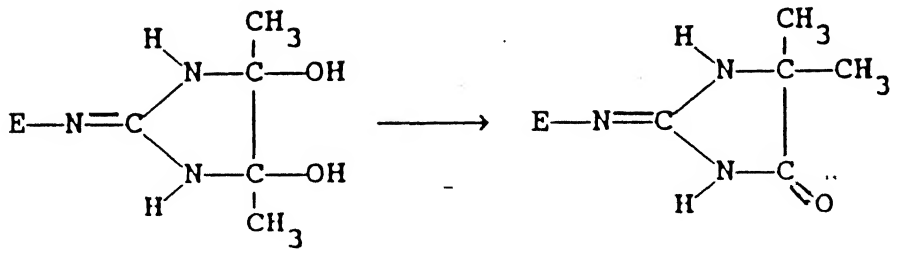
ENZYME PHENYLGLYOXAL

pH 7 - 8

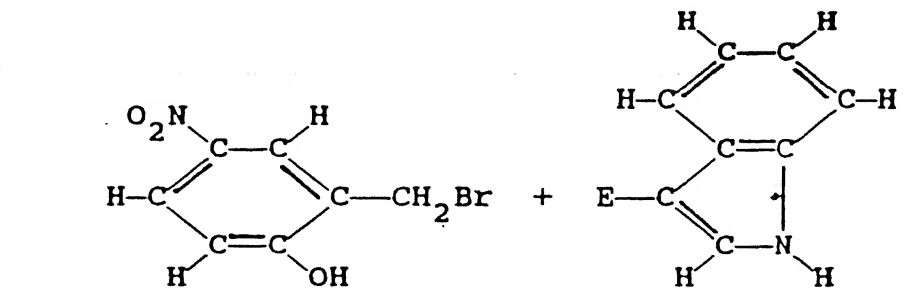


e. Modification of arginine by 2-3 Butanedione

ENZYME 2,3-BUTANEDIONE

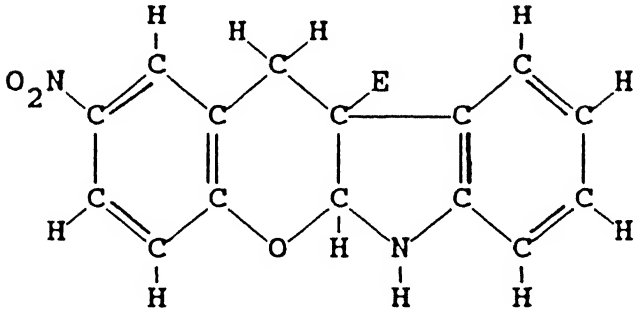


f. Modification of tryptophan by 2- Hydroxy-5-nitrobenzyl bromide



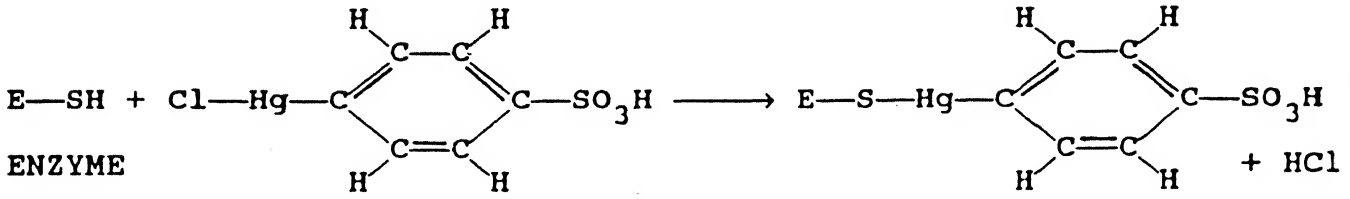
2-HYDROXY-5-NITROBENZYL BROMIDE

ENZYME



+ HBr

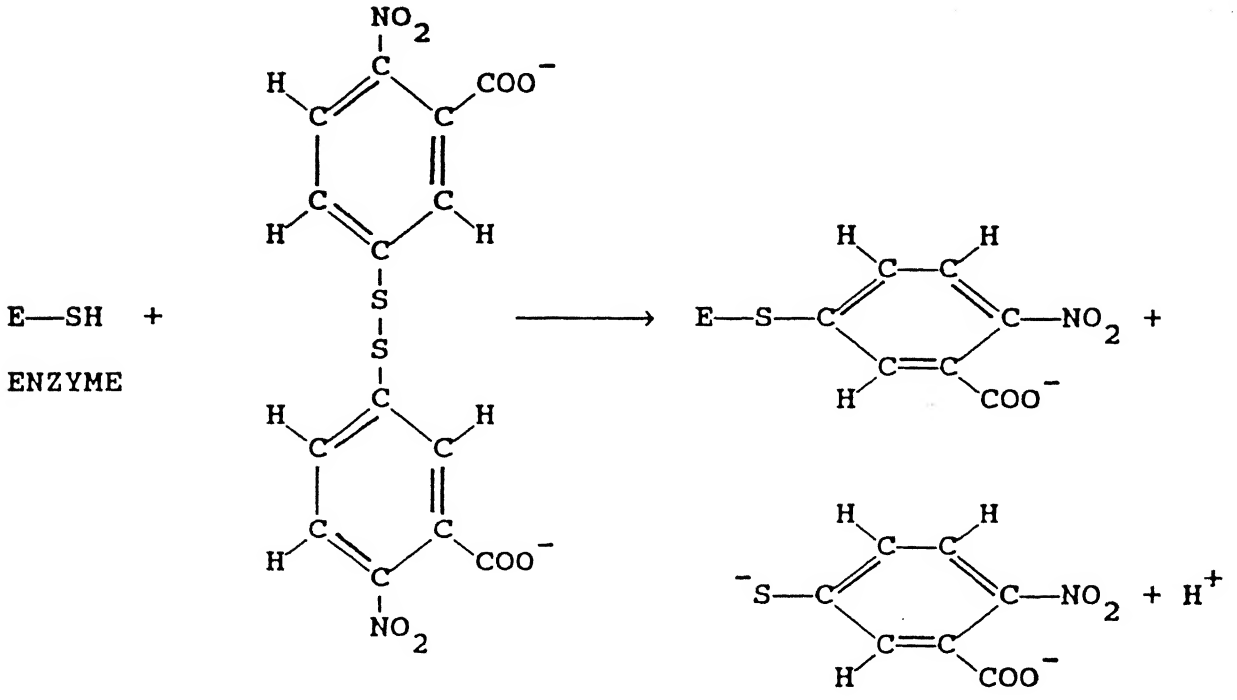
g. Modification of cysteine by p-Chloromercuric phenyl sulfonic acid



ENZYME

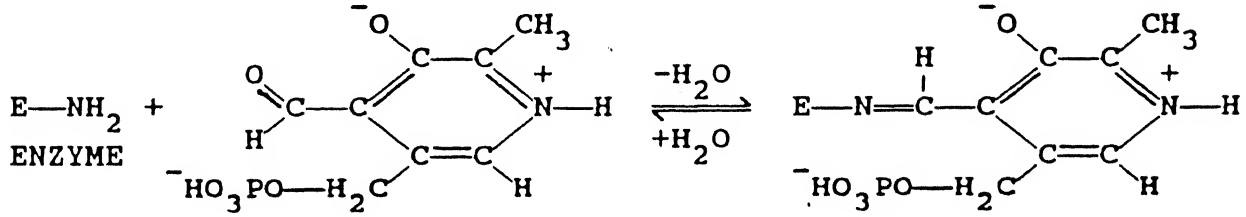
p-CHLOROMERCURIC PHENYL SULFONIC ACID

h. Modification of cysteine by 5,5′-Dithio bis-(2-nitrobenzoic acid)

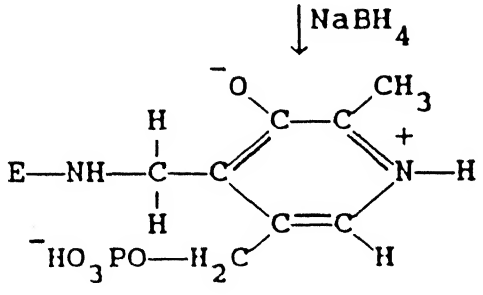


5,5′-DITHIO BIS(2-NITROBENZOIC ACID)

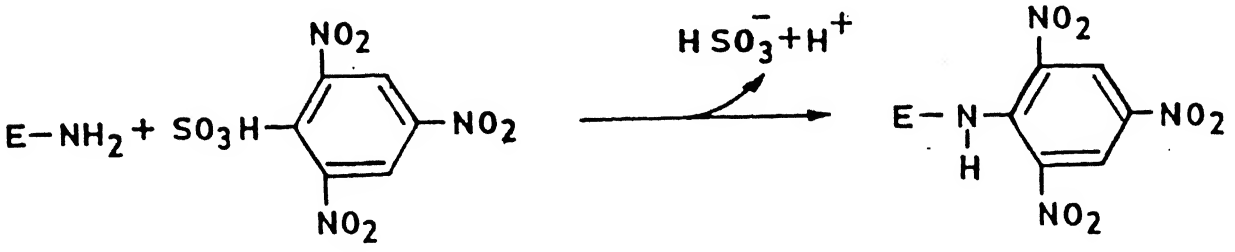
i. Modification of lysine by Pyridoxal-5′-phosphate



PYRIDOXAL-5′-PHOSPHATE



j. Modification of lysine by 2,4,6-trinitrobenzenesulphonic acid



I.4.3.3 *Affinity reagents*

These compounds are chemically reactive analogs of enzyme ligands. Owing to the structural similarity they show affinity towards the ligand binding site (84,85). Therefore, considering affinity labelling in broad sense, one must include all the reactions through which an actual or potentially reactive compound will initially form a reversible enzyme-reagent complex analogous to the enzyme-ligand complex. Once the reagent is bound at the specific site, it will react irreversibly with an amino acid residue present at that site. For example, fluoro sulfonyl benzoyl adenosine (FSBA), is a reagent which reacts specifically with lysine at the nucleotide binding region of the enzymes.

I.4.3.4 *Selectivity*

This refers to the sensitivity of a particular reagent towards the environment surrounding the group(s) it can modify. One with a high degree of specificity is of little use if it

does not show selectivity. Little is understood about the role of a particular type of side chain in enzyme function if all the residues react equally with the reagent. Therefore, if a chemical modifier reacts with high degree of selectivity, then it will modify a specific side chain or several of that type of side chain at different rates. such reagents have been found useful in examining the environment around the reactive functional groups present at the catalytic site.

I.4.3.5 ***Reversibility***

Some of the chemical modifiers bind reversibly to the enzyme but most of them do not (86). This characteristic feature is useful in predicting the nature of active site. The reversibility is also dependent on nature and orientation of the catalytic amino acid residues present at the active site of the enzyme. For example, in malate dehydrogenase the binding of pyridoxal 5'-phosphate, an ε-amino group specific reagent, is not reversed even if the reaction is allowed to proceed for longer time (87). However, in other enzymes complete reversal is obtained [88,89].

I.4.3.6 ***Stoichiometry of reaction***

This is one of the most important properties of a chemical modification, by which, the number of essential amino acid residues involved in the catalytic reaction of enzyme can be determined. Different approaches such as radioactive

labelling [90], amino acid analysis [91] and spectroscopy [92,93] have been used to determine the stoichiometry. Of these, spectroscopic technique is most convenient for determining the incorporation of spectrally active groups. The incorporation of modifier in the enzyme can be co-related with residual activity of the enzyme. The number of amino acid residues critical for the enzyme activity thus can be determined.

I.4.3.7 *Kinetic analysis of modification*

Kinetic investigations of the modification reaction with enzyme provides useful information regarding the mechanism of inactivation process [26,94-96]. The kinetics of enzyme modification reaction depends on the conditions of the reaction system. Usually the inactivation process follows pseudo-first order kinetics (*i.e.* the concentration of the modifier is much higher than that of the enzyme) [81]. The pseudo first order rate constant (k_{obs}) can be calculated from the equation 1.

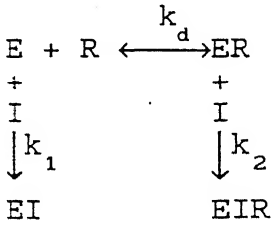
$$-\ln\left[\frac{[E]_t}{[E]_o}\right] = k_{obs}\, t \qquad \text{——— 1}$$

Where, $[E]_o$ and $[E]_t$ represent the enzyme activities at zero and at any given time t, respectively. Plot of

pseudo-first order rate constants against inhibitor concentration gives a straight line and the slope gives the second order rate constant.

I.4.3.7.1 ***Estimation of enzyme-reactant dissociation constant from the inactivation kinetics of reactions involving formation of an intermediary complex***

The kinetic mechanism for the chemical modification of an enzyme can be used for determining the dissociation constant for the binding of ligand to the enzyme [97-100]. This procedure provides valuable information on the binding of substrates or cofactors to the enzymes. A general scheme for this inactivation mechanism in presence of reactant is



Where, both the free enzyme (E) and the enzyme-reactant complex (ER) can react with the inhibitor to produce inactive enzyme inhibitor complex EI and enzyme-reactant complex EIR, with rate constants k_1 and k_2, respectively. The binding of R to E is dependent on the

magnitude of the dissociation constant k_d.

It has been reported by Malebrán and Cardemil [97] that if the inhibitor is capable of binding with enzyme-ligand complex i.e $k_2 \neq 0$, then, only a partial protection in the presence of a reactant is obtained. However, when $k_2 = 0$, total protection is observed against inactivation as saturating concentration of substrate does not allow the binding of inhibitor to the enzyme.

I.4.3.8 *Characterization of enzyme-inhibitor complex*

Binding of an inhibitor to enzyme or protein is usually accompanied by the formation of the complex which can be characterised. The complex gives information regarding the location of reactive residue and nature of the environment around it. The characteristic absorbance of complex formed at a particular wavelength is used in quantitative estimation of inhibitor reacting with enzyme. The absorption maxima and extinction coefficient (ε) of some of the reagents forming characteristic adduct with enzyme are shown in Table I.1.

I.4.3.9 *Location of amino acid residues in tertiary structure*

The concept of exposed and buried residues has been proposed for the degree of accessibility of amino acid residues

to chemical modification. The exposed residues are present on the surface of enzyme whereas, the buried ones are located in its hydrophobic core. When the protein is denatured these buried residues also become accessible to modification. Several techniques have been used to investigate the presence of residues in the interior or exterior regions of the protein.

Table I.1

REAGENT	ABSORPTION MAXIMUM	EXTINCTION COEFFICIENT ($M^{-1}cm^{-1}$)
1. 5, 5'-Dithiobis(2-nitro-benzoic acid) [99]	412 nm	13600
2. p-Chloromercuricphenyl-sulfonic acid [85]	250 nm	7500
3. Pyridoxal 5'-phosphate [85,89]	325 nm	8500
4. Diethyl pyrocarbonate [89]	240 nm	3200
5. Phenylglyoxal [100]	250 nm	11000
6. o-phthalaldehyde [101-104]	337 nm	7660

Ray and Koshland [105,106] proposed a kinetic model to determine the number of residues exposed on the surface and those located in the outer region of the protein. In 1982,

Eftink and Jameson [107] characterized two tryptophan (Trp) residues of alcohol dehydrogenase by fluorescence quenching mechanism. They observed that Trp-15 is exposed to the solvent, whereas Trp-314 is present in the hydrophobic region of the protein. The quenching of oxygen was found to be five times more effective for Trp-15 residue indicating that it is more accessible. These studies supported the kinetic analysis of Ray and Koshland [105] that amino acid residues present in the interior of the proteins are less accessible to a chemical modifier than the exposed ones. These studies were in agreement with the results reported by other workers too [108,109].

I.4.3.10 *Effect of pH on enzyme inactivation*

Inactivation of enzyme by a specific chemical modifier at different pH can be used to determine the pK_a value of a particular functional group involved in the reaction. For example, it is possible to obtain the pK_a value for catalytically important group in an enzyme from kinetic studies, which provides an evidence for confirming the specific aspects of chemical mechanism of an enzyme action. It has been observed that pK_a values of some of the basic amino acids are lower in proteins as compared to the pK_a value reported for free amino acids [110-113]. This difference in the pK_a values has been found to be essential for enzymatic activity as

proteins in general are stable only within a relatively limited pH range, most often near neutrality. Nevertheless, there are many exceptions, e.g. pepsin exhibits the most unusual property since it is stable at acidic pH and gets rapidly inactivated in neutral or alkaline pH.

I.4.3.11 *Use of o-phthalaldehyde, a fluorogenic bifunctional compound in chemical modification studies*

Fluorometry has proven to be a valuable technique in the study of the active site of enzymes [114-119]. A specific chemical modifier forms a fluorescent adduct with the functional group of enzyme accompanied by the loss of catalytic activity. It is essential that fluorescent adduct has absorption and fluorescence properties distinct from that of tyrosyl and tryptophenyl residues of the protein. Fluorescence characteristics can also be helpful in understanding the nature of the catalytic environment because the maxima of the emission spectra is red or blue shifted depending on the characteristic environment present at the active site.

o-Phthalaldehyde, a fluorogenic bifunctional cross linking reagent is amongst very few compounds which have been used for the characterization of nature of active site of the enzyme. The fluorescence property of this compound on reacting with amino acid in presence of reducing agents like

β-mercaptoethanol was first reported by Roth [120]. Benson and Hare [121] identified the product formed by the reaction of o-phthalaldehyde with amino acid and β-mercaptoethanol to be an isoindole adduct having a characteristic fluorescence emission pattern. It was also observed that o-phthalaldehyde forms a non fluorescent adduct with amino groups in the absence of β-mercaptoethanol [122,123]. The use of o-phthalaldehyde as an active site reagent was characterized by Palaczewski et. al. [124]. They showed that aldolase was inactivated when treated with o-phthalaldehyde forming an isoindole derivative by cross linking ε-amino and sulfhydryl groups located at the catalytic site. The mechanism of isoindole formation is shown in Fig I.2.

o-Phthalaldehyde reaction with enzymes is characterized by the absorbance maximum at 337 nm and fluorescence emission maximum at 400-450 nm [103,104,124-128]. Free o-phthalaldehyde does not exhibit any fluorescence over the spectral range 400-500 nm upon excitation at 337 nm [112]. The variation in emission maximum arises due to the difference in the microenvironment of cysteine and lysine residues participating in the formation of isoindole derivative. The molar transition energy is calculated from the fluorescence emission maximum (λ_{em}) of an isoindole derivative using following relationship [124].

$$E_T = 2.985\ \lambda_{em} - 1087.28$$

Plot of λ_{em} (nm) and E_T according to above equation is shown in Figure I. 3. Minimum energy value of 121 kJ/mol was obtained for 1-(β-Hydroxyethylthio)-2-β-hydroxyethylisoindole (EA adduct) in non polar medium of hexane. Maximum E_T was obtained in H_2O, which was more than 250 KJ/mol. On comparing the molar transition energies (E_T) of 1-(β-hydroxyethylthio)-2-β-hydroxy ethyl-isoindole (EA), a synthetic isoindole, in various solvents and the isoindole derivative formed with cysteine and lysine residues of the enzymes, it has been shown that active site of enzymes are relatively hydrophobic in nature. The values of E_T obtained for enzyme-o-phthalaldehyde adduct were close to synthetic isoindole in hexane which indicated the relatively hydrophobic nature at the catalytic center. Besides these interesting features of o-phthalaldehyde which suggested that it acts as an active site specific reagent for many enzymes [103,104,124-128]. It was found that it also binds at the non catalytic site leading to conformational changes and thus resulting in inactivation as observed in octopine and lactate dehydrogenases [129].

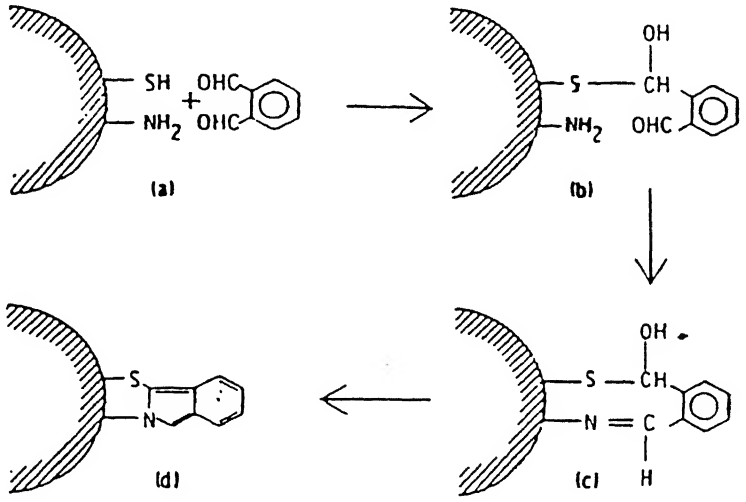


Figure I.2: *Schematic representation of the reaction between O-phthalaldehyde and thiol group of cysteine and ε-amino group of lysine of an enzyme.*

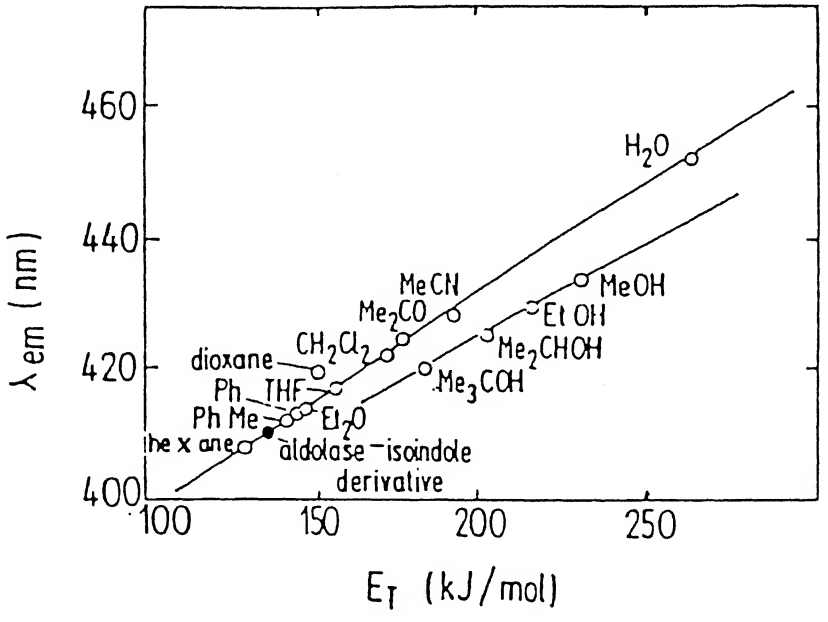


Figure I.3: *Relationship between λ_{max} fluorescence of EA adduct and molar transition energies (E_T) found in different solvents.* Fluorescence maximum of 8μM EA adduct in: n-hexane, tolune (phMe), benzene (ph), ethyl ether (Et_2O). 1,4-dioxane (dioxane), tetrahydrofuran (THF), methyl chloride (CH_2Cl_2), acetone (Me_2CO), acetonitrile (MeCN), tert-butanol (Me_3COH), 2-propanol (Me_2CHOH), ethanol (EtCH), methanol (MeOH) and water (H_2O).

I.5 OBJECTIVE OF THE PRESENT WORK

Fatty acid synthetase from rat liver, adipose and lactating mammary gland, chicken liver, rabbit mammary gland and uropygial gland have many common features. Besides having the same size, charge, shape and phosphopantetheine content the animal fatty acid synthetases have two identical subunits containing seven different component activities for the synthesis of fatty acid on each of its subunit. Although extensive work has been carried out on the mechanism, kinetics, enzymatic activities and properties of seven different component activities, not much information is available on the nature of amino acids at the component enzyme active sites of the enzyme. Chemical modification of enzymes is one of the most effective methods for identification of amino acids which participate in either substrate binding or catalysis. This technique was applied to characterize active site of reductases of fatty acid synthetase. Various chemical modifiers were used to probe the functional groups of essential residues. In the present study it has been shown that lysine residue is involved in the catalysis by chemically modifying the enoyl reductase of FAS with pyridoxal 5'-phosphate and 2,4,6-trinitrobenzene sulphonic acid, the lysine specific reagents. Histidine was

found essential for the enoyl-CoA reductase activity of fatty acid synthetase by chemically modifying the enzyme with diethyl pyrocarbonate. Phenylglyoxal and 2,3 butanedione were used to determine whether arginine is essential for the enzymatic activity. Prevoius studies have shown that sulfhydryl reagents like N-ethylmaleimide, p-chloromercuribenzoate, iodoacetamide, sodium arsenite have inhibitory effects on fatty acid synthesis by blocking acetyl-CoA and malonyl-CoA binding sites. In the present study 5, 5'-Dithiobis(2-nitrobenzoic acid) and iodoacetamide have been used to study their effect on enzyme inactivation and salt induced conformational change. The effect of a bifunctional reagent o-phthalaldehyde was examined as this reagent can bind with -SH and $-NH_2$ groups of cysteine and lysine residues, respectively to give isoindole derivative. The resulting isoindole gave characteristic absorption and fluorescence emission peaks near 337 nm and 400-450 nm, respectively. It also gives an idea of the nature of the environment near the participating -SH and ε-amino groups of cysteine and lysine, respectively. Double inhibition study using o-phthalaldehyde and DTNB was performed to know whether the two inhibitors bind to the same functional group where the blocking of the functional group by one reagent prevents the other from binding to the enzyme molecule.

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CHAPTER II

PURIFICATION CHARACTERISATION AND PROPERTIES OF PIGEON LIVER FATTY ACID SYNTHETASE

II.1. INTRODUCTION

Fatty acid synthetase has been purified from different animal sources using different combination and sequence of techniques like ammonium sulfate fractionation, calcium phosphate gel adsorption, DEAE-cellulose chromatography, sephadex G-100 chromatography. The pigeon liver enzyme was purified to an activity of 300 n moles to 1400 n moles of NADPH oxidised per min per mg of protein using some modifications in the procedures [1-3]. The fatty acid synthetase enzyme system of pigeon liver exists in the soluble portion of the cell as multienzyme complex with the molecular weights in the range 4.5×10^5 to 5.67×10^5 [2,4-6]. The properties of FAS have been extensively studied [3,4]. The enzyme shows maximum activity at an temperature of 25^0C and pH of 6.7. It has been reported that the enzyme is stable in high ionic strength buffer and in low ionic strength buffer it dissociates into half molecular weight

subunit with the loss of synthetase activity. The enzyme can be stored in 0.2 M potassium phosphate buffer containing 1 mM EDTA and 10 mM DTT at -20°C. In the present study fatty acid synthetase from pigeon liver was purified by the method of Muesing and Porter [3] and the effect of ionic strength, pH and temperature were also studied.

II.2 EXPERIMENTAL PROCEDURE

II.2.1 Materials

Well fed (fat free diet) Indian pigeons were used as source for FAS, Acetyl-CoA, Malonyl-CoA, NADPH, $(NH_4)_2SO_4$, $Cacl_2.6H_2O$, $Na_3PO_4.12H_2O$, EDTA, DEAE cellulose, buffer components, were procured from Sigma. Chem. Co, St. Louis, USA. Cysteamine (Fluka), Diketene (Aldrich), DTT (Calbiochem) were purchased from the above designated sources. Reagents used for gel electrophoresis were purchased from Sigma. Chem. Co. and Bio-Rad Lab, Richmond, U.S.A. All other chemicals used were of highest purity grade available commercially.

II.2.2 Methods

II.2.2.1 Production of FAS by pigeons maintained in different nutritional states

Three dozens of healthy pigeons were fed fat free diet for 15 days and divided into four groups. First group was sacrificed as feeding was completed. The second group and third

group were kept on fast for 48 h and refed a fat free diet for 24 h and 48 h respectively. Fourth group of pigeons was fasted for 48 h. before killing. Birds were killed in groups of eight by decapitation and the livers were pooled and chilled immediately.

II.2.2.2 Preparation of crude FAS from pigeons' liver

Preparation of the pigeon liver crude extract was accomplished by the procedure of Wakil et. al [7] and all operations in this procedure were carried out at $0^{o}C$. Immediately following the decapitation of the pigeons, livers were removed, cleaned, and placed on ice. The chilled livers were then homogenized in 1.5 volumes of a phosphate-bicarbonate buffer (70 mM $KHCO_3$), (85 mM K_2HPO_4 and 9 mM KH_2PO_4), (pH 8.0.) containing 1 mM EDTA and 1mM dithiothreitol in a warring blender for 30 s . The homogenate was filtered through a single layer of cheese cloth and centrifuged at 10,000 rpm for 30 min to remove the cell debris, nuclei and mitochondria and the sediment was discarded. The supernatant solution was recentrifused at 35,000 rpm for 90 min. The resulting supernatant solution was stored under nitrogen in sealed cellulose nitrate tubes (40 ml) at $-20^{o}C$. In this state, the fatty acid synthetase was stable for at least 1-2 months.

II.2.2.3 Purification of pigeon liver fatty acid synthetase

Purification was carried out by a sequence of techniques at 25°C.

II.2.2.3.1 *First ammonium sulfate fractionation*

A 40 ml sample of supernatant solution was thawed and warmed to an ambient temperature. Purification was carried out at the temperature (22- 25°). Saturated ammonium sulphate was added dropwise to the crude enzyme solution and stirred gently on a magnetic stirrer under a stream of nitrogen, until a final concentration of 25% saturation was reached. Stirring was continued for an additional 15 min and then the suspension was centrifuged at 10,000 g and 4°C for 10 min. The supernatant was brought to 40 % of saturation with saturated ammonium sulphate, stirred for 15 min and centrifuged again as before. The supernatant solution discarded and the pellet was dissolved in 20 ml of 5 mM potassium phosphate buffer (pH 6.8), and the protein concentration was determined by the method of Gornall et.al [8]. Further purification steps through the calcium phosphate gel and DEAE-cellulose chromatography were followed immediately after the solubilization of enzyme in 5 mM phosphate buffer, because the enzyme is unstable at low ionic strength and gradually dissociates to form inactive half molecular weight subunits. Further the enzyme is also

suceptible to oxidative inactivation at low ionic concentrations when 2-mercaptoethanol is used [9].

II.2.2.3.2 *Calcium phosphate gel adsorption*

The above enzyme preparation and an amount of calcium phosphate gel equal to half of the weight of the protein were simultaneously added to 150 ml of 5 mM phosphate buffer. The calcium phosphate gel solution was prepared by the procedure of Keilin and Hartrec [10].The above mixture was stirred on a magnetic stirrer. The suspension was centrifuged immediately at 4000 g for 3 min. Since, the fatty acid synthetase is not stable in a medium of low ionic strength, the next step in the purification procedure, DEAE-cellulose chromatography was followed immediately.

II.2.2.3.3 *DEAE-cellulose chromatography*

A column of DEAE-cellulose (10.3x3.5 cm) was prepared, from material that has been prewashed and equilibrated with approximately 400 ml of 40 mM phosphate buffer. The flow rate of column was kept at 5 ml/min. Dithiothreitol may be omitted from the first portion of the wash. The gel-treated enzyme solution was applied to the column, and the latter was washed with 40 mM phosphate buffer until the absorbance of the eluate, at 280 nm, is less than 0.05. Elution was carried out with 0.25 m phosphate buffer. A single protein peak emerged from the column after about 60 ml of eluate volume. An additional 30-40

ml of eluate was collected.

II.2.2.3.4 ***Second ammonium sulfate fractionation***

Saturated ammonium sulfate was added to the eluate obtained from DEAE-cellulose chromatography to 26% of saturation with stirring under an atmosphere of nitrogen. The suspension was centrifuged as before and the precipitate was discarded. The supernatant solution was brought to 32% saturation with saturated ammonium sulfate, stirred and centrifuged. The precipitate obtained after centrifugation contained active enzyme which was dissolved in about 1 ml of 0.2 M phosphate buffer (pH 6.8), containing 1 mM DTT. After this stage of the procedure the preparation usually has very less amount of contaminating proteins and the preparation was used for most studies after dialysis against 0.2 M phosphate buffer. DTT (10 mM) was added to the preparation recovered after dialysis and the preparation was then divided into aliquots which was stored frozen at -20^{o}C. Before use, the enzyme solution was thawed and then kept at room temperature for at least 30 min. This equilibration is essential, since the rapid inactivation of enzyme occurs at 0^{o}C [9].

II.2.2.4 Gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the standard method of Laemmli [11] with certain

modifications. Gel slabs of size 138x130x2 mm contained 5% (w/v) acrylamide for resolving gel and 4% (w/v) for stacking gel. To the enzyme sample, 4 volumes of sodium dodecyl sulfate buffer (pH 6.8) containing sodium dodecyl sulfate, 2%, Tris-HCl, 62.5 mM, glycerol, 1% (v/v),5% (w/v) 2-mercaptoethanol and 0.05% bromophenol blue was added and heated in a boiling water bath for 2 min prior to electrophoresis. Electrophoresis was carried out with a current of 2 mA per lane for 6-8 h. The protein bands were fixed with solution containing acetic acid (5%, v/v) and (10% ,v/v) methanol for 15 min, stained for 1h with 0.25% (w/v) coomassie brilliant blue and destained by repeated washing in 7% (v/v) acetic acid.

II.2.2.5 Assay of the overall FAS activity

The overall activity for purified fatty acid synthetase was measured by following the rate of decrease of NADPH concentration spectrophotometrically at 340 nm according to the method of Muesing and Porter [3]. The reaction mixture contained 100 μM malonyl-CoA, 33 μM acetyl-CoA, 100 μM NADPH 0.2 M potassium phosphate buffer (pH 7.0), 1mM EDTA, 1mM DTT and 5-10 μg of purified fatty acid synthetase complex, in a total reaction volume of 1.0 ml. The reaction was carried out by the addition of enzyme to the mixture of substrates previously equilibrated at the desired temperature for 10 min.

The concentrations of NADPH, acetyl-CoA and malonyl-CoA stock solutions were determined using extinction coefficients of $6.22 \times 10^3 cm^{-1}M^{-1}$ at 340 nm, $15.4 \times 10^3 cm^{-1}M^{-1}$ at 259 nm and $14.6 \times 10^3 cm^{-1}M^{-1}$ at 260 nm, respectively. Absorbance was measured at 25°C in UV-VIS spectrophotometer, (Shimadzu, Model UV-160A). Specific activity is expressed as nanomoles of NADPH oxidised per minute per milligram of protein. Division by 14 converts the value to nanomoles of palmitate formed per minute per milligram of protein.

II.2.2.6 Protein determination

The concentration of protein in crude preparation was determined by the biuret method using bovine serum albumin as standard [8]. Concentration of purified enzyme protein was determined from the absorbance at 280 nm using 3.87×10^5 $M^{-1}cm^{-1}$ as the molar absorption index [5]. The molecular weight of the protein was taken as 450,000.

II.2.2.7 Calculation of specific activity of enzyne

The specific activity of enzyme is expressed in Units/mg of protein. One unit of the enzyme is defined as the amount of enzyme that will form one n mole of palmitate per minute.

By Beer-lambert Law:

Absorbance, $A = \varepsilon cl$ or $c = \frac{A}{\varepsilon l}$

Where ε = extinction coefficient

c = concentration in moles/litre

l = path length

$\varepsilon^{NADPH} = 6.2 \times 10^3$ litre mol^{-1} cm^{-1} at 340 nm and l = 1 cm.

$$c = \frac{A_{340/min}}{6.2 \times 1000 \times 1} \text{(mole/litre) } min^{-1}$$

$$c = \frac{A_{340/min}}{6.2} \text{ } (\mu \text{ mole/ml}) \text{ } min^{-1}$$

$$c = \frac{A_{340/min} \times 1000}{6.2} \text{(n moles/ml) } min^{-1}$$

$$\text{Hence, specific activity} = \frac{A_{340/min} \times 1000}{6.2 \times \text{mg enzyme/ml reaction mixture}}$$

Specific activity is expressed here as nanomoles NADPH oxidised per minute per milligram of protein. Division by 14 converts the value to nanomoles of palmitate formed per minute per milligram of protein.

II.2.2.8 Preparation of model substrates and assay of enoyl-CoA reductase and β-Ketoacyl reductase activity

II.2.2.8.1 *Preparation of substrates*

II.2.2.8.1.1 *Synthesis of S-Acetoacetyl-N-acetylcysteamine and S-Crotonyl-N- acetyl cysteamine*

N-acetylcysteamine was prepared from acetyl chloride and

cysteamine as per the procedure described by Martin et. al [12]. For both the substrates N-acetylcysteamine was used. N-acetylcysteamine was synthesised by slowly adding to one mole of acetyl chloride in cold dry chloroform, a solution containing two moles of β-mercaptoethylamine. The chloride salt was filtered off and the evaporation of chloroform from the filtrate yielded an oil. Freshly prepared product was used in all experiments, since thiazoline is formed on standing. The synthesised N-acetyl cysteamine was characterised by NMR. spectroscopy.

II.2.2.8.1.1.a *Synthesis of S-acetoacetyl-N-acetylcysteamine*

S-acetoacetyl-N-acetylcysteamine was synthesized by the method of Lynen and Wieland [13]. To a solution of 10 g of N-acetylcysteamine in 10 ml of ether, cooled in an ice bath, was added slowly a solution of 7.05 g of diketene in 10 ml of ether. The reaction begins at low temperature but is not complete until the mixture is brought to room temperature and allowed to stand for several hours. Upon subsequent cooling to -10°C the oily reaction mixture solidified to a crystalline paste. The solution was filtered and the residue was washed with cold ether. Several recrystallizations from ether led to the pure product in the form of colorless leaves or needles with melting point of 60°C. The compound was characterised by NMR spectroscopy.

II.2.2.8.1.1 b ***Synthesis of S-crotonyl-N-acetylcysteamine***

S-crotonyl-N-acetylcysteamine was synthesized by the method of Kass et. al [14]. N-Acetylcysteamine(8.4 moles) in water (10 ml) was added dropwise to a solution of lead acetate (8.4 moles) in water (10 ml), cooled to 0^{o}C. A heavy yellow precipitate of the lead salt of N-acetylcysteamine was formed immediately. After 30 min it was filtered, washed repeatedly with water and dried in a vacuum desicator over calcium chloride. The crotonyl chloride (0.5 mmole)(prepared by treatment of the crotonic acid with excess thionyl chloride in 1:10 ratio) and lead N-acetylcysteamine (0.2 mmole) were refluxed in benzene (1 ml) for 1 h. During reflux, the color of suspension gradually changed from yellow to white. The reaction mixture was diluted with benzene and filtered. The benzene solution was washed successively with 5% $NaHCO_3$ and 5% NaCl, dried over $MgSO_4$ and evaporated.The thioester was further purified by column and thin layer chromatography. After purification it gave a single spot on thin layer chromatography in 2% methanol in methylene chloride and had absorption bands at 225 mμ and 263 mμ. The yield was calculated from the 263 mμ absorption by using ε of 6700 [15]. The product formed was characterised by NMR spectroscopy.

II.2.2.9 Assay of the reductase activity

β-ketoacyl reductase activity was measured by the decrease in NADPH concentration at 340 nm. The reaction mixture contained 100 μM NADPH, 14.8 mM S-acetoacetyl -N-acetylcysteamine (as model substrate) and 5 μg of purified enzyme in a total volume of 1.0 ml. Similarly, enoyl-CoA reductase activity was measured by the oxidation of NADPH at 340 nm and the reaction mixture contained 100 μM NADPH, 13.5 mM S-Crotonyl-N-acetylcysteamine (as model substrate) and 1 mg purified enzyme in a total reaction volume of 1.0 ml.

II.2.2.10 Effect of ionic strength, pH and temperature on FAS activity

To study the effect of ionic strength on the stability of FAS the purified enzyme (2 mg/ml) was incubated with various concentrations (1-200 mM) of potassium phosphate buffer (pH 7.0), containing 1 mM EDTA and 1 mM DTT at 25^{o}C. Aliquots were withdrawn immediately (for initial enzyme activity) and at different time intervals for the residual enzyme activity determination using the assay mixture previously described in section II.2.5. Similar procedure was followed for studying the effect of pH on FAS stability except that the enzyme was incubated at constant ionic strength of 0.2 M potassium

phosphate buffer with different pH values (6.0-8.0) at 25°C.

Effect of temperature on the rate of inactivation of the fatty acid synthetase complex was studied by incubating the enzyme (2 mg/ml) in the reaction mixture containing 0.2 mM potassium phoshate buffer pH 7.0, 1 mM EDTA and 1 mM DTT at different temperatures (0°C-37°C). Aliquots were removed at different time intervals and assayed in the assay mixture as previously described in section II.2.5. An aliquot was withdrawn for initial enzyme activity determination after addition of the enzyme to the buffer maintained at required temperature.

II.3 RESULTS AND DISCUSSION

II.3.1 Production of FAS by pigeons maintained in different nutritional states

Lipogenic enzymes of mammalian liver are known to be markedly affected by nutritional and hormonal stress [16]. Thus, it has been shown that a reduction in the activity of liver glucokonase [17], acetyl coenzyme A carboxylase [18,19], glucose 6- phosphate dehydrogenase [20,21], long chain acyl-CoA desaturase [22] and the citrate cleavage enzyme [23,24] as well as the soluble fatty acid synthetase [20,25,26] accompany fasting of the animal. The activities of these enzymes are returned to normal values upon refeeding and in certain cases may rise to levels far above normal if fat free diets are used

Table II.1

Fatty acid synthetase activity in the soluble fraction of the livers of Fed, Starved, and Refed pigeons

Status of birds	Liver weight (gm)	Supernatant Fraction			
		Total soluble protein (mg)	Total Activity (Units)	Specific* activity (Units/mg)	Units/gm liver
Fed	4.2	227	417.6	1.84	99.4
Starved (48 h)	3.9	194	75.7	0.39	19.4
Refed (24 h)	5.0	225	428	1.9	85.6
Refed (48 h)	5.5	235	475	2.0	86.4

*Specific activity are expressed as nanomoles of palmitate formed per minute per mg of protein.

in realimentation [20,27].

Fatty acid synthetase enzyme of the liver homogenate supernatant fraction of the pigeons maintained in different nutritional states have shown variation in total protein content, total FAS activity and specific activity Table II. 1. The data in Table II. 1 are given as average values per bird, within a given nutritional state. It is evident from the

Table II. 1 that there is no significant difference between the liver weights of the fed and the starved birds. However, the liver weight of refed birds were significantly higher than those of fed and starved birds. When the crude supernatant fraction was assayed for *de novo* fatty acid synthesis, it was found that enzyme activity (specific activity or total units) was decreased by a factor of about five on starvation. Although the total amount of protein isolated from the liver supernatant of the starved birds did not differ markedly from the fed birds, but the total enzyme units from fed birds were much higher than those obtained from the starved birds. This may be due to the presence of very little of the intact fatty acid synthetase complex present in the protein isolated from starved birds. The activity per gram of liver after refeeding was lower, which might be because of an increase in liver weight, presumably due to an increase in glycogen and water content.Similar results were reported by Butterworth et.al [26] in experiments with pigeons maintained in different nutritional status except. that the values of the liver weight, total protein and specfic activity found were higher than the present data. This might be due to some environmental and genetic factors imposed on Indian pigeons. Appreciable quantities of other protein species may also be present in the supernatant fraction of the starved bird's liver. The recovery of enzyme activity in refed birds occurs through adaptive enzyme

synthesis. Hicks. et. al. [28] have shown that rise in the level of rat liver fatty acid synthetase upon refeeding can be prevented by administration of puromycin or actinomycin D at the beginning of the refeeding period. Their results suggest that the increase in FAS activity on refeeding is due to de novo synthesis of enzyme protein and not due to activation of preexisting enzyme. The net amount of enzyme present in any given state is a function of both synthetic and degradative parameters and it was found that it is the rate of synthesis and not that of degradation, that is the major parameter in controlling the liver FAS in rats subjected to nutritional stress. However in the early stages of fasting a change in the degradative parameter may also be important [29].

II.3.2 Purification of pigeon liver FAS

The preparation of pigeon liver supernatant was carried out using the birds of 48 h refeeding and the data is shown in Table II. 2 and the results of subsequent purification of this preparation are shown in Table II. 3. Pigeon liver supernatant fraction, prepared in 40 ml volume has protein concentration of 24 mg/ml and 2.89 U/mg of the specfic activity which was increased in the next step to ~7.5 U/mg by ammonium sulfate fractionation resulting 2.6 fold of purification with an overall yield of 88%. Further purification of this fraction through a DEAE-cellulose column resulted in the increase of specific activity to 28 U/mg with an overall yield of 26%.

Further ammonium sulfate fractionation to 26-32% of saturation, 12 fold purification was obtained with specific activity of 35 U/mg. The specific activities reported here in different steps of purification for crude enzyme extracted from liver of refed birds Table II. 2 and 3 are lower than those reported by Muesing and Porter [3]. The specific activity of 35 U/mg, obtained after the 26-32% of ammonium sulfate fractionation was approximately half of the value obtained by Muesing and Porter [3]. It is not possible to establish completely the reasons for the above discrepancies. However, several factors are there which can affect the activity of an enzyme preparation for fatty acid synthesis. A factor that may affect the activity of a crude extract of pigeon liver for fatty acid synthesis is the nutritional status of the bird.

Table II.2

Preparation of pigeon liver supernatant solution

Fraction	Vol (ml)	Total Activity (Units)	Total Protein (mg)	Specific Activity (Units/mg)	Yield %
Homogenate	140	10,716	11,524	0.93	—
10,000xg	100	6313	3322.5	1.89	49
35,000xg	80	4856	1886	2.57	36

Table II.3.3

Purification of pigeon liver Fatty acid synthetase

Fraction	Volume (ml)	Total activity Units	Total protein (mg)	*SA (Units/mg)	Yield	Purification %
Pigeon liver supernatant	40	2733	943	2.89		
$(NH_4)_2SO_4$ (25-40%)	15	2411	320	7.5	88	2.59
DEAE cellulose	20	700	25	28	25.6	9.6
$(NH_4)_2SO_4$ (26-32%)	1.5	542.5	15.5	35	19.8	12.1

*SA = Specific activity is expressed as nanomoles of palmitate formed per minute per mg of protein.

We have also observed that the specific activity of the enzyme decreased to 15 U/mg for the birds, those were in 24 h of refeeding. As only the preliminary studies are available on the effect of this factor on FAS activity of pigeon liver, it is not possible to have an exact conclusion. A second factor affecting the fatty acid synthetase activity of pigeon liver may be the environmental factor which is applicable to Indian

pigeons.

The purified enzyme obtained after successive steps of purification was homogeneous and showed a single band of 225 kDa per subunit of FAS on SDS-polyacrylamide gel electrophoresis.

II.3 3 Effect of ionic strength, pH and temperature

It has been suggested earlier that attainment of proper orientation of the individual enzymes of fatty acid synthetase complex for optimum enzyme activity requires phosphate ions [30,31]. Therefore, stability of the complex at pH 7.0 in the presence of different concentrations of phosphate buffer was examined. The rates of enzyme inactivation at different phosphate concentrations were found to differ markedly (Fig. II. 1). After 1 h 50% of the original enzyme activity was lost in 1 mM phosphate, whereas in 40 mM phosphate, no appreciable change in activity occurred. A characteristic feature of inactivation rate profiles in 1 to 5 mM phosphate buffer was the nonlinearity of the plot of the log of percentage of initial activity against the time. At higher phosphate concentrations the rates of enzyme inactivation were not followed for sufficient periods of time to allow a judgement of the linearity of the semi-logarithmic plots. The biphasic inactivation rate profiles suggest that the active complex changes to modified active species which then undergo

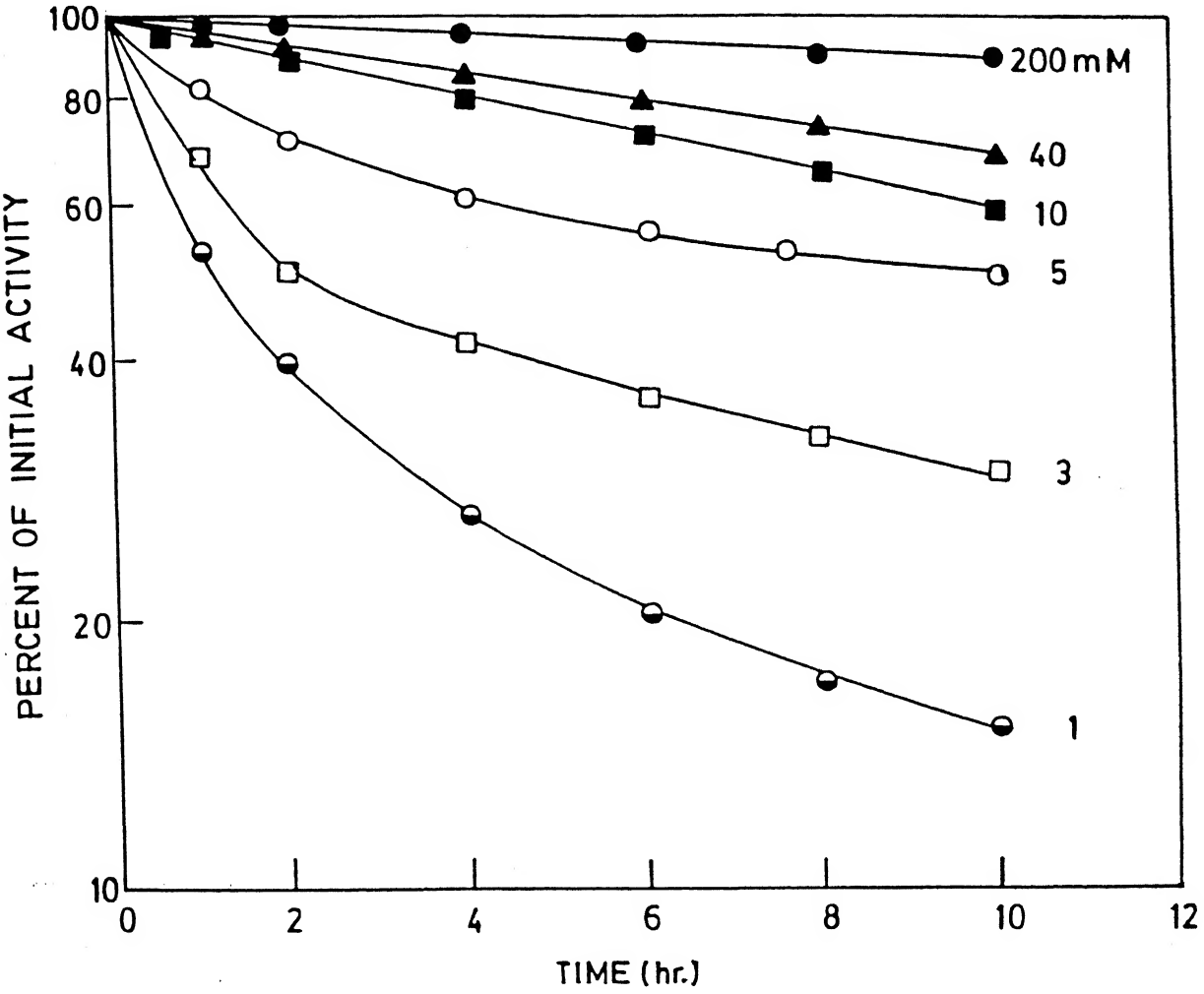

Figure II.1: Semilogarithmic plots of the rate of inactivation of the fatty acid synthetase complex at pH 7.0 and 25°C in various concentration of phosphate buffers containing 1 mM EDTA and 1mM DTT.

inactivation and dissociation to subunits.

The maximum stability of fatty acid synthetase at neutral pH was previously recognised [4]. We have measured the rate of inactivation of pigeon liver enzyme at various pH values, at a constant ionic strength (Fig. II. 2). At pH values lower than 6.5 a sharp decline in enzyme activity occurred. At pH 6.0, 90% of the original enzyme activity was lost in about 4 h. The loss of FAS activity at lower than pH 6.5 may be attributed in part to aggregation and precipitation of the protein as it nears its pI value of 5.6. On alkaline side of neutrality the inactivation rate increased with the increase in pH.

Polymeric structures stabilized by hydrophobic interactions tend to dissociate more readily as the temperature is lowered below the room temperature. The rate of inactivation of FAS at various temperatures are shown in (Fig. II. 3). The rate of inactivation is highest at $0^{o}C$ and then slows as the temperature is raised. In the temperature range 18-$25^{o}C$, the inactivation rate was the lowest and increased as the temperature is raised beyond $30^{o}C$. The time taken for the loss of half of the original activity was calculated to be 2 h at $0^{o}C$ and this occurs due to the weakened hydrophobic interactions. The increased inactivation rate above $25^{o}C$ cannot, however be explained on the basis of weakened hydrophobic interactions. The explanation for this result may lie in the disruption of hydrogen bonds and in the

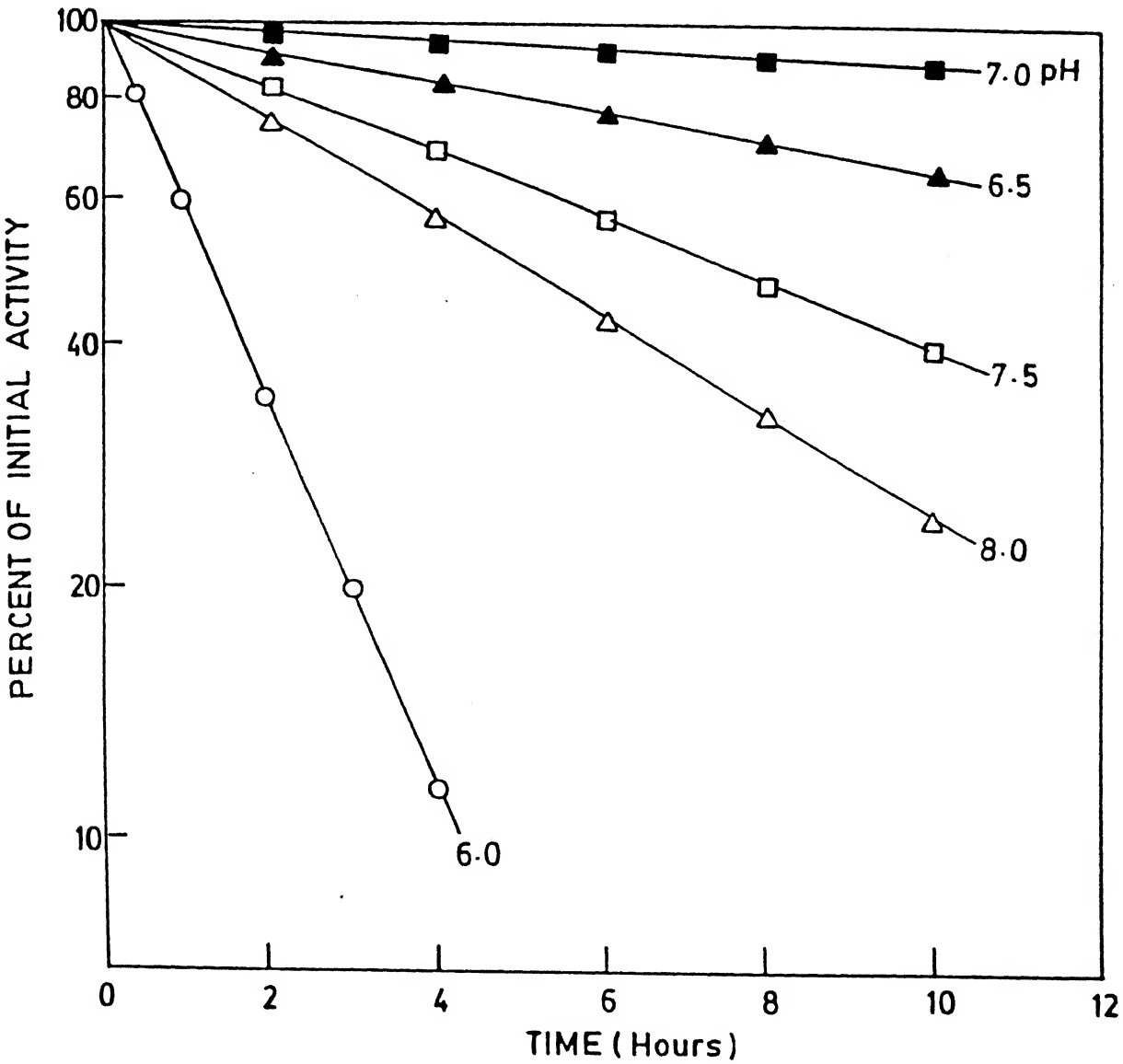

Figure II.2: Rate of inactivation of fatty acid synthatase at 25°C and a constant ionic strength of 0.2 M potassiun phosphate buffer containing 1 mM EDTA and 1 mM DTT as a function of pH.

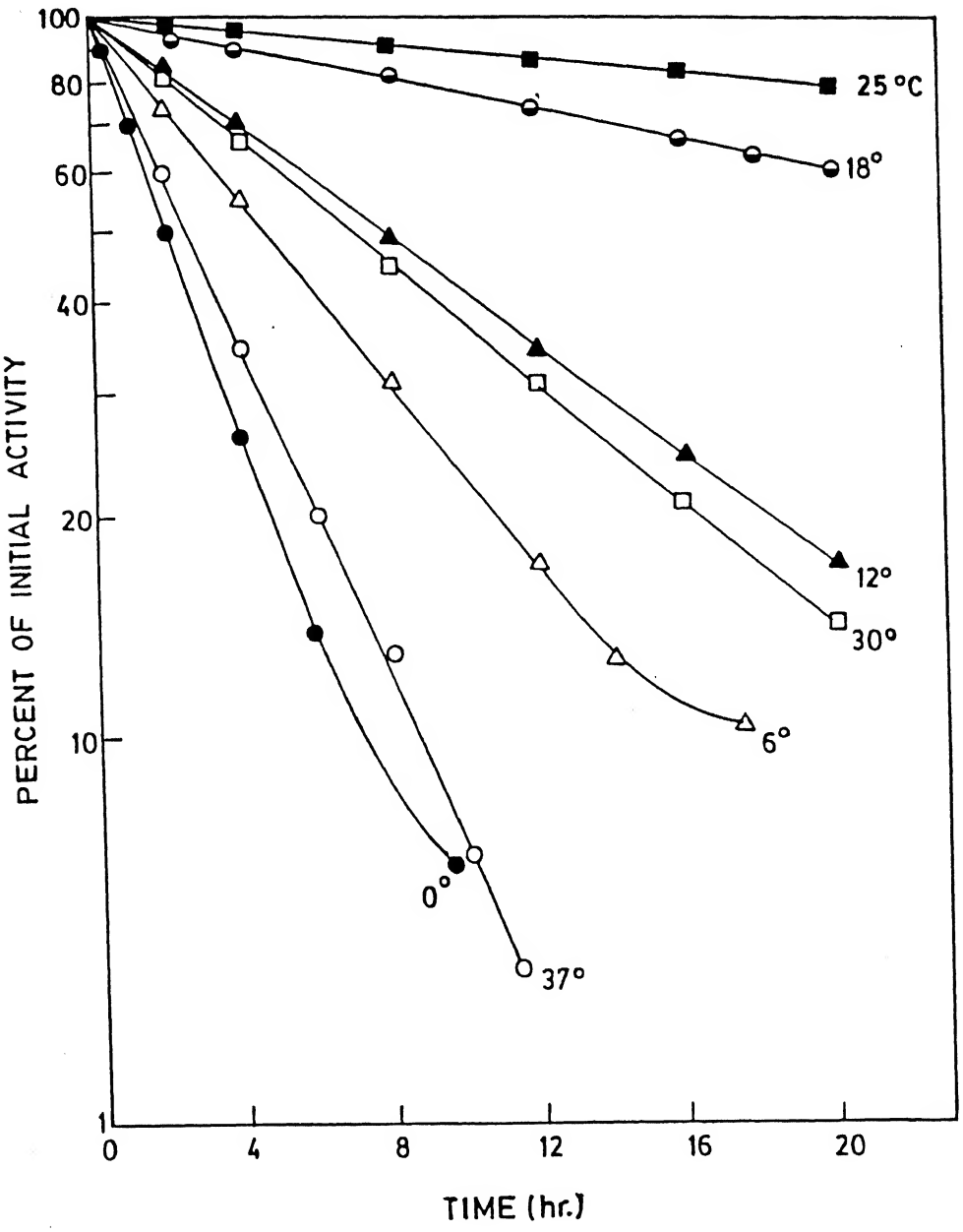

Figure II.3: Effect of temperature on the rate of inactivation of the fatty acid synthetase complex in 0.2 M potassium phosphate buffer pH (7.0) containing 1 mM EDTA and 1 mM DTT.

strengthening of electrostatic repulsive forces at higher temperatures. It was observed that there is considerable activation of the rate of fatty acid synthesis in the assay medium when the enzyme is inactivated at a low temperature ($0^{o}C$ and $6^{o}C$) and then assayed at $30^{o}C$ in 0.2 M potassium phosphate, pH 7.0. This might be due to the fact that a portion of subunits which can be easily activated through reassociation, to form active complex for sudden change in temperature.

II.4 Summary

In this chapter purification, characterization and properties of pigeon liver FAS have been described. Pigeons were maintained in different nutritional states. It was found that the specific activity of FAS in the supernatant fraction extracted fron starved pigeon's liver was markedly lower than that of refed pigeons. There was a marginal increase in the specific activity of refed pigeon as compared to the fed pigeons. The liver weight also varied with the nutritional states. The enzyme was purified by $(NH_4)_2SO_4$ fractionation, calcium phosphate adsorption and DEAE cellulose chromatography. After purification by the different sequential steps the specific activity of the supernatant fraction of the refed pigeon's liver extract increased from 2.89 U/mg to 28 U/mg with an overall yield of 25 % resulting in more than 9 fold of

purification. In the last step of purification by ammonium sulphate fractionation the specific activity was increased to 35 U/mg with 12 fold purification giving approximately 20 % of overall yield. The purified enzyme was characterized by SDS polyacrylamide gel electrophoresis, that showed a single band corresponding to homogeneous preparation of enzyme of molecular weight 225,000. The above values of specific activity were less than the values reported previously. This difference might be due to environmental factors and some other factors viz. genetic and nutritional states affecting the Indian pigeons. The effects of ionic strength, pH and temperature on the rate of inactivation of the enzyme were also studied. With the variation of ionic strength of buffer, pH and temperature of the incubation medium it was found that the FAS of pigeon liver is most stable in the high ionic strength buffer at pH of 7.0 and at a temperature of 25^{o}C.

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CHAPTER III

IDENTIFICATION OF ESSENTIAL LYSINE AND HISTIDINE RESIDUES PRESENT AT THE ENOYL-CoA REDUCTASE DOMAIN OF PIGEON LIVER FATTY ACID SYNTHETASE USING CHEMICAL MODIFIERS

III.1 INTRODUCTION

Pigeon liver fatty acid synthetase is a dimeric enzyme consisting of two half molecular weight multifunctional polypeptide chains which contain the seven component enzyme activities and the ACP site with its 4'-phosphopantetheine prosthetic group [1-6]. The synthesis of long chain fatty acids from acetyl-CoA and malonyl-CoA involves numerous sequential reactions and acyl intermediates covalently bound to protein [7-9]. FAS has two NADPH dependent reductase activities, viz. enoyl-CoA reductase and β-ketoacyl reductase. Enoyl-CoA reductase of FAS catalyses the following reaction:

$$CH_3CH{=}CHCOS\text{-}ACP + NADPH + H^+ \longrightarrow CH_3CH_2CH_2COS\text{-}ACP + NADP^+$$

Although extensive work has been carried out on the mechanism

of fatty acid synthesis, not much information is available on the nature of amino acid residues present at the active site. Chemical modification of pigeon liver FAS with pyridoxal 5'-phosphate (PLP) in dark results in specific modification of lysine residue with the loss of enoyl-CoA reductase activity [10]. Recently, chemical modification studies with chicken liver FAS using diethyl pyrocarbonate has shown that an essential histidine residue is present at the active site of the enoyl-CoA reductase [11]. Lysine has been identified as the catalytic residue in various enzyme systems using the lysine specific reagents PLP [10,12-15] and 2,4,6-trinitrobenzene sulphonic acid (TNBS) [16,17]. It has been reported that PLP can act as photosensitizing agent, in the reaction of enzymes with PLP upon irradiation with visible light, which involves light-activated addition of a nucleophilic residue to the pyridoxal 5'-phosphate-lysine azomethine bond from a second proximal base [18-20]. Imidazole groups of histidine have been identified as the nucleophilic residues in close proximity of lysine in several studies of photosensitization of enzymes by PLP [18,19,21-23]. The present study has shown the presence of lysine and histidine residues at the active site of enoyl-CoA reductase of pigeon liver FAS. Presence of a nucleophilic residue in close proximity of lysine residue has also been

identified by PLP-sensitized photooxidation.

III.2 EXPERIMENTAL PROCEDURE

III.2.1 Materials

2,4,6-trinitrobenzenesulphonic acid (TNBS), Pyridoxal 5'-phosphate (PLP), Diethyl pyrocarbonate (DEPC), Acetyl-CoA, Malonyl-CoA, NADPH, 2'-AMP, 2',5'-ADP, 5'-AMP, Imidazole, N-Acetylhistidine, 5,5'-dithiobis-(-2-nitro benzoic acid) (Sigma), Sephadex G-25, Sephadex G-50 (Pharmacia), Dithiothreitol (DTT) (Calbiochem) were obtained from designated sources. All other chemicals were of highest purity grade commercially available. Distilled water from Millipore Milli Q system was used in all experiments.

III.2.2 Methods

III.2.2.1 Enzyme preparation and activity measurements

Pigeon liver fatty acid synthetase was purified and assayed spectrocphotometrically by the method of Muesing and Porter [24] and has been described in Chapter-II section 2.3 and 2.5. The enzyme was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the standard method of Laemmli [25] that exhibited a single band.

Assays for β-ketoacyl and enoyl-CoA reductase activities were carried out spectrophotometrically as described earlier [5,26]. Fatty acid synthetase activity was measured by the decrease in the absorbance of NADPH at 340 nm on UV-vis spectrophotometer (Shimadzu, Model UV-160A). All measurements were carried out at 25°C.

III.2.2.2 Calculation of specific activity of the enzyne

The specific activity of the purified enzyme was 35 U/mg of protein which was used in all the experiments. One unit of the enzyme is defined as the amount of enzyme that will form one n mole of palmitate per min. Calculations are described in detail in chapter II section 2.7.

III.2.2.3 Modification of FAS with pyridoxal 5'-phosphate in dark and in light conditions

Inactivation of FAS was carried out at 25°C in 0.2 M potassium phosphate buffer (pH 7), 1 mM EDTA and 1 mM dithiothreitol at a protein concentration of 1.68 mg/ml with various concentrations (0.25 to 5 mM) of PLP. The incubation mixtures were protected from light with aluminium foil.

Photoinactivation was carried out with 1 mM PLP under the same experimental conditions as used in the dark reaction in

the presence of 10 mM dithiothreitol to protect the enzyme from loss of activity under the oxygen atmosphere, required for photoxidation. The sample was irradiated with white light as described by Hathaway and Noltman [18] and kept closed by a rubber stopper with a tubing through which aliquots were withdrawn and water saturated oxygen was introduced into the space above the reaction mixture. Controls in absence of PLP were run in light and in dark under the same experimental conditions. At specific time intervals, aliquots of the reaction mixture were removed and assayed immediately for overall FAS activity and enoyl-CoA reductase activities.

III.2.2.4 Effect of substrates on the inactivation of FAS by PLP

The enzyme was first incubated with substrates for 15 min follwed by the addition of PLP. All the incubation mixtures prepared for these experiments contained enzyme, substrate, and PLP in a total volume of 100 μl. Similarly in photoinactivation of FAS, protection experiment was performed by incubating the enzyme with NADPH for 15 min prior to addition of PLP under the photomodification condition. After 15 min of incubation aliquots were withdrawn immediately to measure the remaining activity.

III.2.2.5 Reduction of fatty acid synthetase-pyridoxal 5'-phosphate complex with sodium borohydride

Fatty acid synthetase (2 mg/ml) was incubated with 1 mM PLP in 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT at 25°C for 15 min in the dark condition. The reduction of the Schiff's base was then carried out by adding a drop of octyl alcohol followed by sodium borohydride solution (final concentration 10 mM). The reaction mixture was kept at 0°C for 15 min and then passed through a small column of sephadex G-50 according to the method of Penefsky [27] to remove excess reagent before taking the spectra. To prepare the column, a disposable 1 ml plastic tuberculin syringe was plugged with glass wool and filled to the 1 ml calibration mark with sephadex G-50 fine, previously swollen in the reaction buffer. The column was placed in a test tube, allowed to stand until no further liquid drained from it and then it was centrifuged at 900 rpm for 2 min. A sample of reaction mixture (50-100 μl) was transferred to the top of the sephadex column and centrifugation was repeated as before, using a clean test tube to collect the effluent.

III.2.2.6 Reactivation of FAS after inactivation by pyridoxal 5'-phosphate

Reactivation of PLP treated fatty acid synthetase was performed by dilution experiments. FAS enzyme (4 mg/ml) was incubated with 1 mM PLP for 15 min, and the aliquots were withdrawn and added to the assay mixture for determining the residual activity. The incubatuon mixture was then diluted 80 fold with same buffer. The aliquots were withdrawn at different time intervals and the activity of enzyme was determined. Another sample of enzyme was similarly treated and reduced with 10 mM $NaBH_4$ prior to dilution. Similarly, the photomodified enzyme was diluted 80 fold and simultenously the light was switched off and oxygen flow was stopped. Controls were run concurrently in the dark and light conditions in absence of PLP.

III.2.2.7 Spectral analysis of pyridoxal 5'-phosphate modified FAS in the dark and light conditions

The enzyme (1 μM) was incubated with PLP (1 mM) for 15 min. The reaction mixture was treated with sodium borohydride (10 mM final concentration) and incubated at $0^{o}C$ for 15 min. The resulting N^{ε}-phosphopyridoxyllysine complex was characterized by absorption and fluorescence emission spectrum

with excitation at wave length, 325 nm [28]. Similarly, in the light condition the enzyme (2 mg/ml) was incubated with 1 mM PLP for 60 min under the photo-reaction condition and without the reduction by $NaBH_4$. The reaction mixture was passed through sephadex G-50 column to remove the excess reagent. The fluorescence emission and excitation spectra of FAS were recorded at excitation wavelength and emission wavelength of 325 and 445 nm, respectively. A similar spectra was recorded for the sample which was not irradiated. Fluorescence and absorption spectra were recorded using a cuvette of 1 cm path length on Perkin-Elmer Fluorometer (Model LS 50B) and UV-vis spcetrophotometer (Shimadzu, Model UV-160A), respectively.

III.2.2.8 Stoichimetry of the reaction of pyridoxal 5'-phosphate with FAS

Fatty acid synthetase (2 mg/ml) was incubated with 1 mM PLP for 15 min at 25^{o}C in the presence and absence of 5 mM NADPH and subsequently reduced with 10 mM sodium borohydride. The reaction mixture was kept at 0^{o}C for 15 min. After reduction the reaction mixture was passed through G-50 sephadex column [27] to remove the excess reagent. After filtration protein concentration was measured by the method of Lowry et.al

[29]. The number of moles of PLP incorporated per mole of enzyme was determined by taking the ratio of concentrations of N^{ε} phosphopyridoxyllysine and enzyme. The concentration of N^{ε} phosphopyridoxyllysine was determined using an extinction coefficient of 9700 M cm^{-1} at 325 nm[28]. The molecular weight of FAS was taken as 450,000.

III.2.2.9 pH dependence studies of pyridoxal 5′-phosphate inactivation of FAS

Effect of pH on inactivation by PLP was observed over a pH range of 6.0-8.0 using 0.2 M potassium phosphate buffer. Enzyme (1.68 mg/ml) was incubated with 1 mM PLP in 0.2 M potassium phosphate buffer containing 1 mM EDTA and 1 mM DTT. Appropriate controls were also run under similar pH conditions. The effect of pH on enzyme inactivation was analyzed according to the equation 1 [30]

$$\frac{1}{k} = \left[\frac{1}{k_{max}.K}\right] [H^+] + \frac{1}{k_{max}} \qquad (1)$$

Where, k is the second order rate constant at each pH, k_{max} is the maximal second order rate constant, K is the ionization constant of the group involved in the catalysis and $[H^+]$ is the

hydrogen ion concentration.

III.2.2.10 Reaction of FAS with 2,4,6-trinitrobenzenesulphonic acid

As TNBS also reacts with the thiol compounds, the enzyme was passed through sephadex G-50 column to free DTT and $(NH_4)_2SO_4$ before each experiment since these compounds interfere with the TNBS reaction. The enzyme prepared by this method was free from detectable contamination of these compounds and was fully active. In control experiments, it was found that removal of the thiol compound did not affect the enzyme activity. Inactivation was carried out in 0.2 M potassium phosphate buffer (pH 7.0) containing, 1 mM, EDTA with different concentrations of TNBS (0.25 to 2 mM) at the protein conentration (2 mg/ml). At different time intervals, aliquots from the reaction mixture were removed and assayed immediately, for overall FAS and enoyl-CoA reductase activities. Controls without TNBS were run concurrently. Solutions of TNBS were stored in the dark at $0^{\circ}C$ to minimise the hydrolysis. Solutions of NADPH was prepared fresh daily. Protection experiments were performed under similar experimental conditions by incubating the enzyme with substrates for 15 min prior to the addition of TNBS.

III.2.2.11 Measurement of thiol content of 2,4,6-trinitro-benzenesulphonic modified enzyme

The thiol content of enzyme was determined by the method of Ellman [31]. Enzyme solution was freed of DTT by passing it through a sephadex G-50 column previously equilibrated with buffer. Fatty acid synthetase (2 mg/ml) was incubated with 1 mM TNBS for 15 min. Portions (0.25 ml) of the reaction mixture were removed and immediately passed through a sephadex G-25 syringe column to free the enzyme from excess reagent according to the method of Penfesky [27]. The eluted sample was mixed with 0.75 ml portions of 8 M urea solution in 0.2 M potassium phosphate buffer (pH 7.0) containing 0.3 mM EDTA and 20 mM 5,5'-dithiobis-(2 -nitrobenzoic acid). The total concentration of thiol groups in the enzyme was determined by the increase in absorbance at 412 nm over a period of 60 min using the extinction co-efficient of 13,600 $M^{-1}cm^{-1}$.

III.2.2.12 Stoichiometry of reaction of 2,4,6-trinitro benzenesulphonic acid with FAS

The stoichiometry of inactivation by TNBS was determined by incubating FAS (4 mg/ml) with 1 mM TNBS and modification was followed by absorbance increase at 367 nm [32]. The number of moles of TNBS incorporated per mol of enzyme was determined by

h as L-arginine hydrochloride and NADH from yeast were cured from Sigma Chemical Company USA. Sodium pyruvate was chased from Boehringer Mannheim GmbH, Germany.

The chemical modifiers, p-chloromercuricphenyl sulfonic d [PCMS], 5, 5'-dithiobis -(2-nitrobenzoic acid) [DTNB], thylmaleimide [NEM] iodoacetamide and phenylglyoxal were ained from Sigma Chemical Company U.S.A.. The thiol taining reagents like dithiothreitol, glutathione, and ercaptoethanol were also procured from Sigma Chemical pany, USA. All other chemicals used were of highest purity de commercially available. Throughout the experiments quartz ble distilled water was used.

.2.2 Methods

.2.2.1 *Enzyme activity measurements*

Octopine dehydrogenase activity was determined ctrophotometrically using a Gilford spectrophotometer (Model)) equipped with a Gilford recorder. All the components of reaction mixture were prepared in 100 mM potassium sphate buffer (pH 7.0) and their concentrations in assay cture were as follows: 4.8 mM L-arginine, 2.4 mM pyruvate, 14 mM NADH in a total vol. of 1 ml. The reaction was

taking the ratio of concentrations of trinitrophenyl derivative of amino group of lysine (ε-TNP-lysine) and the enzyme. The concentrations of ε-TNP-lysine and the enzyme were determined by using absorbance coefficient of 11,000 $M^{-1}cm^{-1}$ at 367 nm and Lowry et.al, method [29], respectively. The molecular weight of FAS was taken as 450,000. Simultaneously in another set of experiment, aliquots were withdrawn at different time intervals to assay the overall FAS and enoyl-CoA reductase activities.

III.2.2.13 Modification of FAS with diethyl pyrocarbonate

Chemical modification of pigeon liver FAS was performed by reacting the enzyme with the histidine reagent diethyl pyrocarbonate (DEPC). Solutions of nucleotides and DEPC were prepared daily before use. The modification reagent was diluted in ethanol and quantitated as ethoxyformyl imidazole at 240 nm, using a molar extinction coefficient of $3.2x10^3$ $M^{-1}cm^{-1}$ [33]. The reagent is unstable in aqueous medium and decomposes into ethanol and carbon dioxide. The previous results have howed that higher stability is obtained at acidic pH and at low temperature in potassium phosphate buffer [11]. The rate constant, K', for the decomposition of DEPC was determined by incubating the reagent in (0.2 M potassium phosphate buffer containing 1 mM EDTA and 1 mM DTT. Aliquots at appropriate time

intervals were withdrawn added to a large excess of imidazole. The formation of ethoxyformyl imidazole was quantitated at 240 nm. The quantity of reactive DEPC at any time, t, was quantitated and the rate constant of decomposition was determined from a plot of $\log([DEPC]_t/[DEPC]_0)$ versus time. This value of K' was used in all the calculations pertaining to DEPC inactivation. Since, FAS is optimally stable in high ionic strength phosphate buffer at neutral pH, the inactivation experiments were carried out at 0°C in the presence of 0.2 M potasium phosphate buffer (pH 7.0) containing 1 mM DTT and 1 mM EDTA at the enzyme concentration of 3 μM with 5-25 μM concentration of DEPC. At specific time intervals, aliquots from the reaction mixture were removed and assayed immediately, for overall and enoyl-CoA reductase activities. Controls without DEPC in presence of ethanol were run simultaneously.

III.2.2.14 Reversal of diethyl pyrocarbonate inhibition of FAS with hydroxylamine

The enzyme (3 μM) was incubated with DEP (6 μM) for 30 min at 0°C in 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT. To the above reaction mixture 90 mM neutralized hydroxylamine was added and the incubation was continued for an additional 60 min at 25°C and aliquots were

assayed for the enzyme activity. A control was run under similar conditions without hydroxylamine.

III.2.2.15 Spectral analysis of FAS modification by diethyl pyrocarbonate

The enzyme (1 μM) was incubated with (6 μM) of diethyl pyrocarbonate for 1, 5, 10 and 15 min in 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT. The difference spectra were recorded on UV-vis spectrophotometer (Shimadzu, Model-160A) at the indicated time intervals taking untreated enzyme in the reference cell and treated enzyme in the sample cell. Spectra of enzyme were also recorded before and after the treatment with DEPC for 15 min using buffer as blank.

III.2.2.16 Effect of pH on the inhibition of FAS by diethyl pyrocarbonate

Effect of pH on inactivation of FAS by diethyl pyrocarbonate was studied by incubating the enzyme (3 μM) with 6 μM DEPC in 0.2 M potassium phosphate buffer containing 1 mM EDTA and 1 mM DTT over a pH range, 6.0-8.0. Appropriate controls were also run under similar pH conditions. The effect of pH on enzyme inactivation was analyzed following the method

described in the Section III.2.2.9.

III.2.2.17 Spectrophotometric quantification of diethyl pyrocarbonate reaction with FAS

Fatty acid synthetase (8μM) was incubated with diethyl pyrocarbonate (16 μM) in 0.2 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT at 0^{o}C.respectively at 0^{o}C. Ethoxyformylation of hisitidne residues was monitered at 240 nm using an extinction coefficient of $3.2x10^{3}$ $M^{-1}cm^{-1}$ [33]. At time intervals, samples were withdrawn and diluted in 50 mM neutralised N-acetylhistidne to terminate the reaction, and assayed for synthetase activity. The number of reactive histidine residues was determined by monitoring activity loss as a function of ethoxyformylation at 240 nm.

III.3 Results and discussion

In the present work we have probed lysine and histidine as essential residues involved in the catalytic activity of enoyl-CoA reductase of fatty acid synthetase. Various chemical modifiers were used to establish the involvement of lysine and histidine residues.

III.3.1 Inactivation of FAS by pyridoxal 5'-phosphate

Incubation of fatty acid synthetase with PLP resulted in the time dependent loss of synthetase and enoyl-CoA reductase activities. Both the activities were decreased rapidly up to about 8 min and subsequently, the rate of inactivation was slower; and equilibrium was reached after 10 min, as there was little further change in these two activities. The extent of inactivation increased with increasing concentration of PLP (Fig. III.1&2). The time course and the concentration dependence of inactivation as well as the extent of inactivation were quite similar for both the enoyl-CoA reductase and overall activity of the FAS. This observation suggests that inactivation of FAS is due to the modification of an essential lysine residue in the enoyl-CoA reductase domain. Modification of lysine residue present in the enoyl-CoA reductase with PLP has also been reported for goose uropygial gland FAS [34]. The rate of inactivation was higher as compared to the pigeon liver FAS. Inactivation studies of yeast FAS with PLP have shown that modified lysine is present in the β-ketoacyl reductase and not in the enoyl-CoA reductase [13].

The determination of the rate of modification of an enzyme by an inhibitor as a function of pH allows one to calculate the pk_a of the reactive group in an enzyme. The inactivation

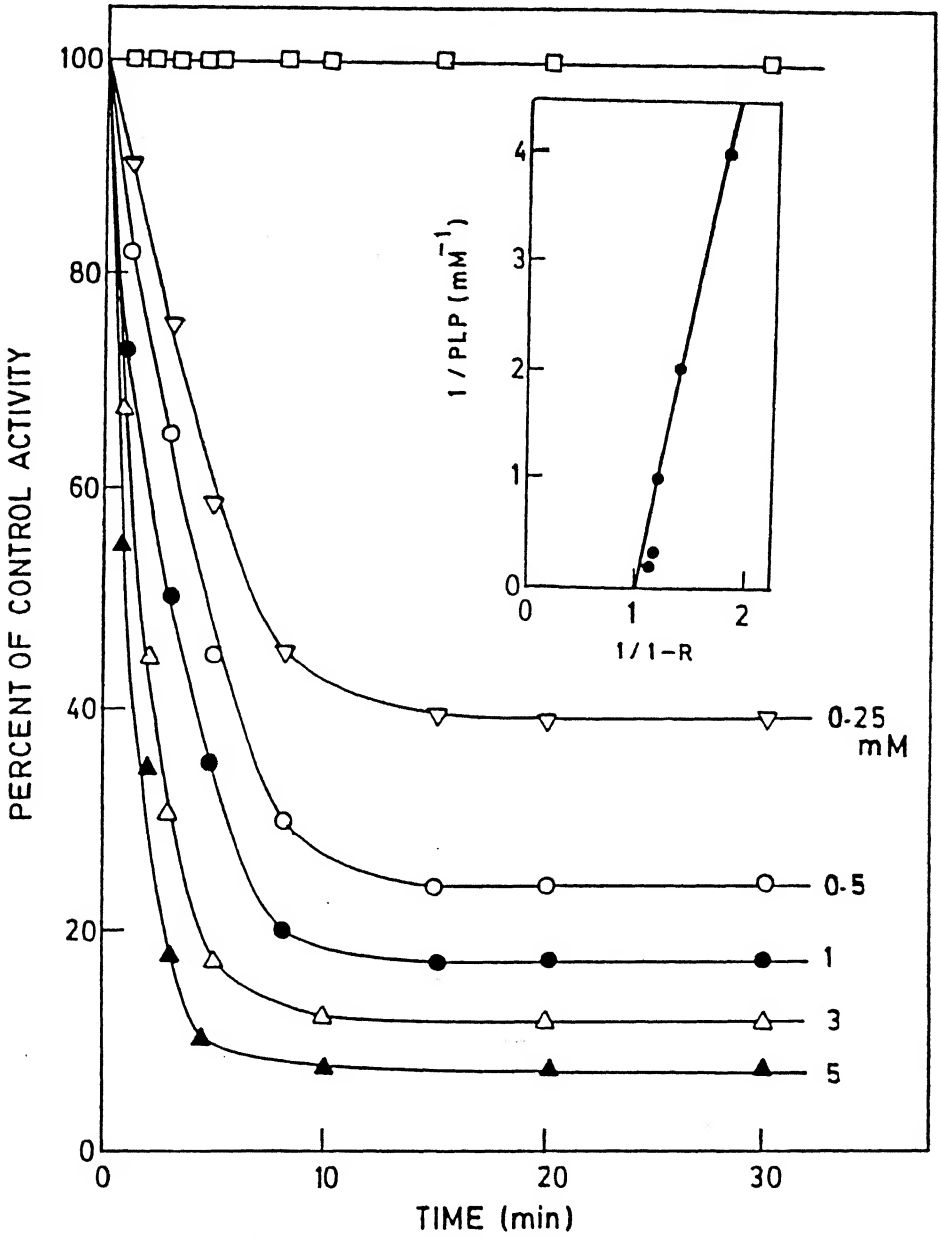


Figure III.1: *Time dependent inactivation of overall fatty acid synthetase activity by PLP.* The enzyme (1.68 mg/ml) in 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT with 0.25 (▽), 0.5 (o), 1 (●), 3(Δ), and 5 mM (▲) PLP at 5°C. Aliquots were withdrawn at the indicated time intervals and assayed the residual activity as described in the Experimental Procedure". Inset: a double reciprocal plot of fractional inactivation against pyridoxal phosphate concentration (R, fractional residual activity).

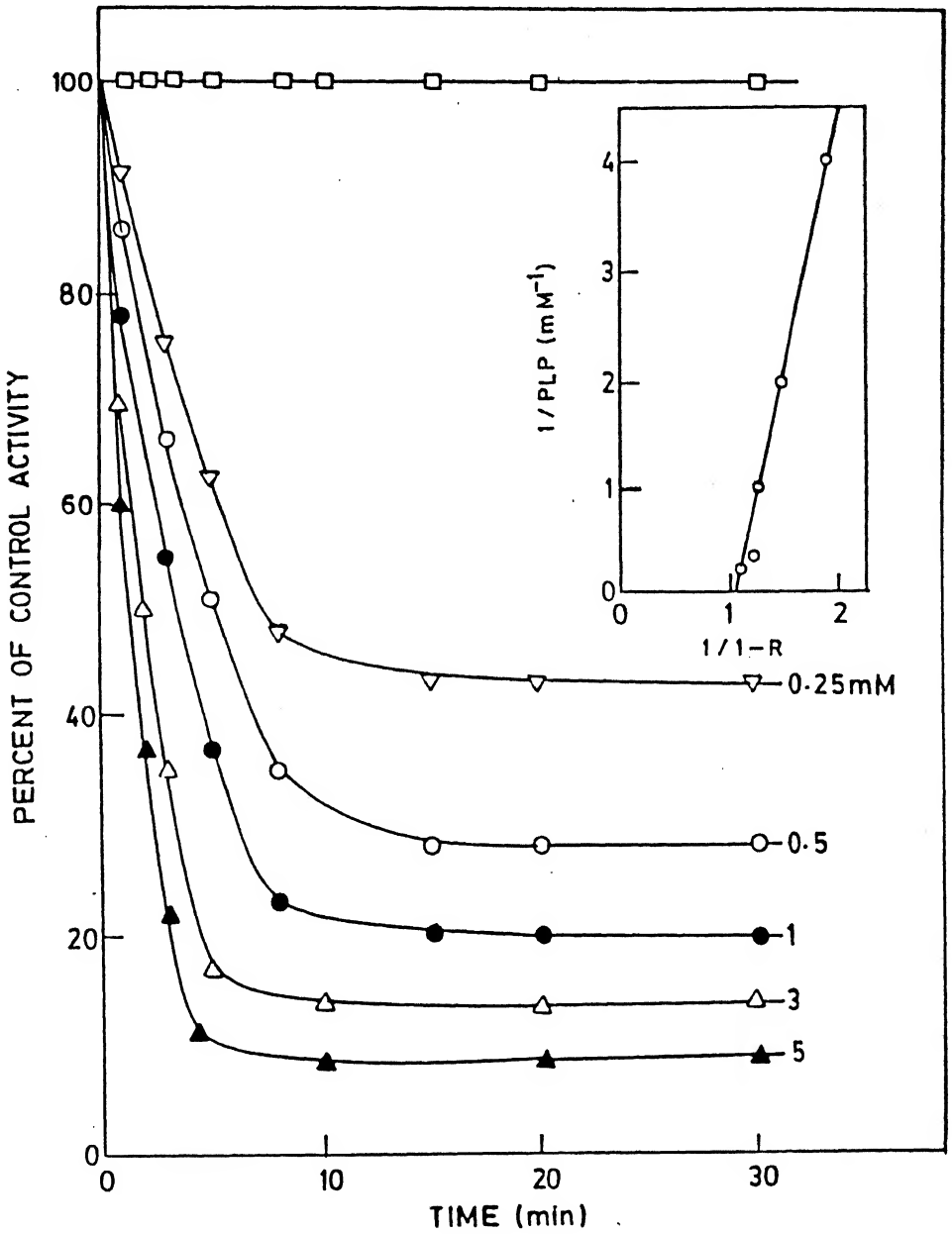


Figure III.2: *Time dependent inactivation of enoyl-CoA reductase activity by PLP.* The enzyme (1.68 mg/ml) in 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT with 0.25 (▽), 0.5 (o), 1 (●), 3(Δ), and 5 mM (▲) PLP at 25°C. Aliquots were withdrawn at the indicated time intervals and assayed the residual activity as described in the "Experimental Procedure". Inset: a double reciprocal plot of fractional inactivation against pyridoxal phosphate concentration (R, fractional residual activity).

pattern of FAS by PLP in the pH range of 6.0-8.0 is shown in (Fig. III 3). A plot of log of percent control activity *vs* time gave a straight line. The apparent second order rate constants obtained as described in section 2.2.9 were plotted as a function of pH (Fig. III 3 inset a). The data from the linear plot was used for the determination of pk_a of the reacting residue in PLP that is shown in (Fig. III.3 inset b) and the pk_a value of 6.8 was obtained, indicating that the micro environment of lysine modified by PLP is hydrophobic in nature. The hydrophobicity renders the ε-amino group of lysine to remain in deprotonated form which is essential for Schiff's base formation between lysine and PLP.

III.3.2 Kinetic analysis of the inactivation of FAS with pyridoxal 5'-phosphate

It was found that treatment of the enzyme with apparently saturating concentrations of pyridoxal 5'-phosphate did not result in the complete inactivation and a small amount of overall activity and enoyl-CoA reductase activity was always remained, just as previously observed with modification of other enzymes with this inhibitor [35-37]. To test whether this residual activity is due to unreacted native enzyme or partially active modified enzyme, the data were subjected to

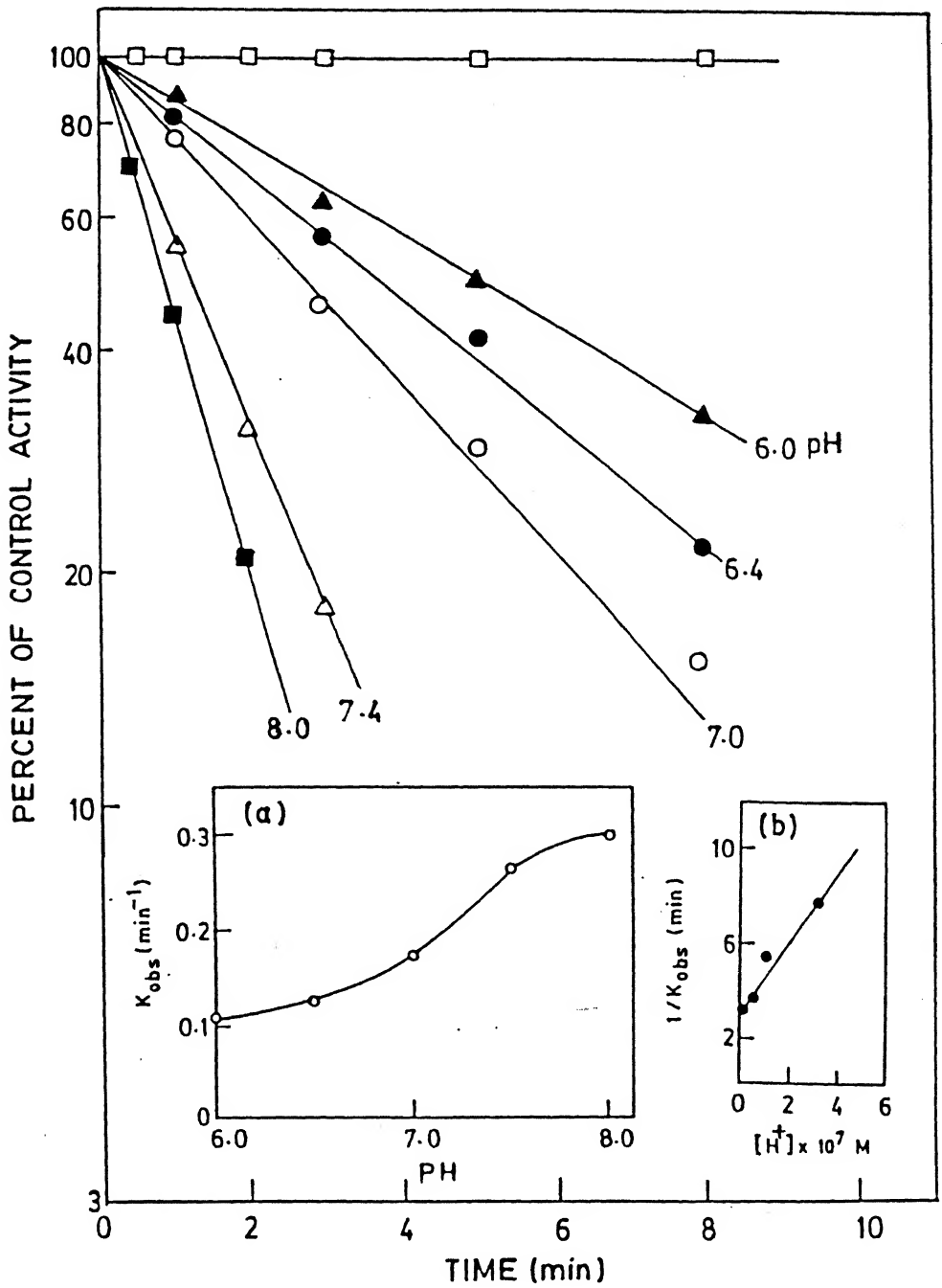


Figure III.3: *Fatty acid synthetase inactivation by PLP as a function of pH.* The enzyme (1.68 mg/ml) was inactivated with 1 mM PLP in 0.2 M potassium phosphate buffer over a pH range of 6.0-8.0. At indicated time the aliquots were removed and enzyme activity was determined. Inset **(a):** Plot of dependence of pseudo-first order rate constant of inactivation on pH. Inset **(b):** $1/K_{obs}$ min *vs* $[H]^+$ reploting the data of (A)].

kinetic and equilibrium analysis as described by Chen and Engel [38]. In this model, the residual activity is due to the equilibrium between catalytically inactive Schiff's base and a noncovalent enzyme-pyridoxal phosphate complex, according to the following scheme of Chen and Engel [38],

$$E + PLP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E.PLP \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\text{-}PLP$$

Scheme-I

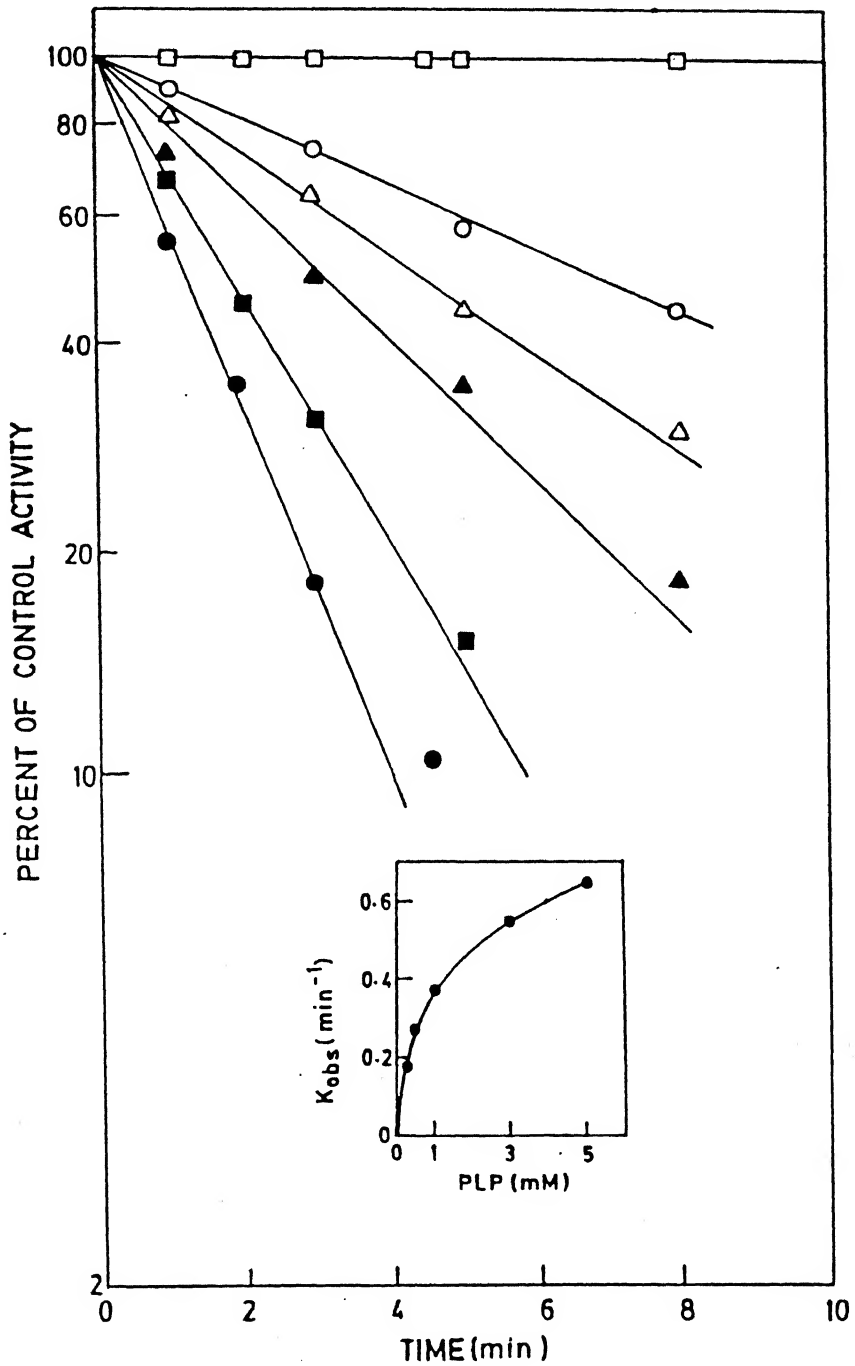
where, E.PLP is the noncovalent complex and E-PLP is the Schiff's base formed between the enzyme and the reactant. The noncovalent binding step is rapid in both the directions and dissociates almost quantitatively to yield free active enzyme in the assay mixture. The first order interconversion of noncovalent E-PLP complex to Schiff's base is slow in both the directions. k_4 is a finite rate constant and its contribution to the pool of active enzyme can be neglected as normal assay time for the enzyme is only 60 s. The initial inactivation thus represents the rate of formation of covalent complex (k_3) and is given by the following relationship,

$$k_{app} = \frac{[\text{pyridoxal-P}]k_3}{[\text{pyridoxal-P}] + k_1} \quad \text{---------eq(1)}$$

Where, $k_1=k_2/k_1$ and k_4 is assumed to be negligibly small as compared to k_1 and k_2. Taking reciprocals of eq (1),

$$\frac{1}{k_{app}} = \frac{1}{[\text{Pyridoxal-P}]} \frac{k_1}{k_3} + \frac{1}{k_3} \quad \text{---------eq(2)}$$

This relationship indicates that the double reciprocal plot of k_{app} versus PLP should be linear with a slope of k_1/k_3 and intercept of $1/k_3$. The data in Fig III. 1&2 were subjected to pseudo-first order kinetic analysis. The reaction of overall and enoyl reductase activity with PLP followed pseudo-first order kinetics as indicated by the linearity of semi-log plots of residual activity vs time (Fig. III.4&5). The plot of pseudo-first order rate constants versus PLP concentration gave a hyperbola (Fig III.4&5 inset) and reciprocal plot of pseudo-first order rate constant and PLP concentration yielded a straight line which did not pass through the origin as shown in (Fig.III.6 a&b). These results are consistent with the idea that a kinetically significant enzyme-PLP complex is formed prior to inactivation. The values of 0.59 mM and 0.59 min^{-1} for the dissociation constant $k_1=(k_2/k_1)$ and k_3 respectively were obtained from the intercept and slope of the reciprocal plot. Almost similar values were obtained from the Enoyl-CoA reductase activity plot. According to the reaction scheme 1,



gure III.4: *Kinetics of inactivation of overall fatty acid nthetase by PLP.* The enzyme (1.68 mg/ml) in 0.2 M potassium osphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT th 0(□), 0.25 (○), 0.5 (Δ), 1 (▲), 3(■), and 5 mM (●) PLP at °C. Aliquots were withdrawn at the indicated time intervals d assayed the residual activity as described in the xperimental Procedure". The inset shows a plot of observed eudo-first order rate constant *vs* initial PLP ncentrations, from which a second order rate constant was tained.

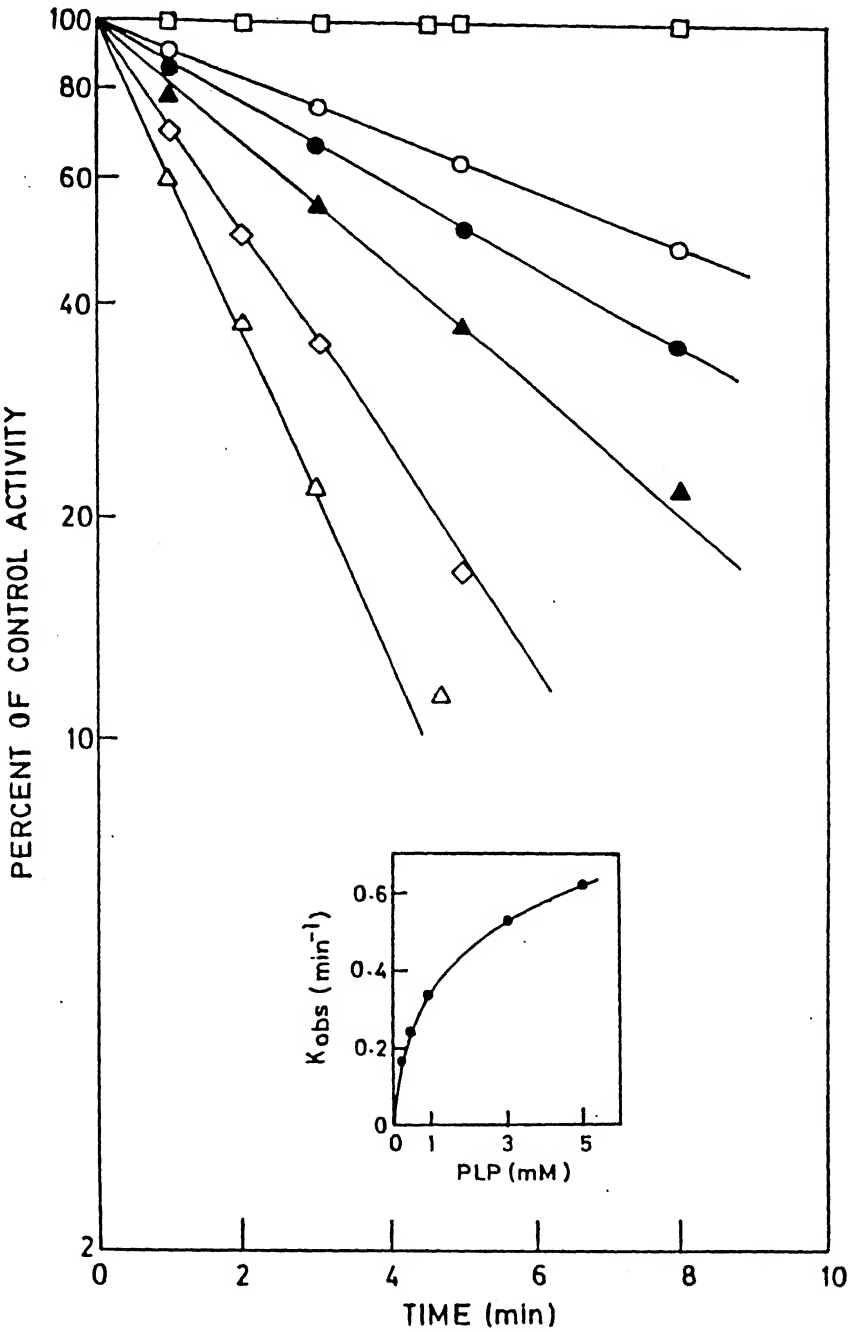


Figure III.5 *Kinetics of inactivation of enoyl-CoA reductase activity by PLP.* The enzyme (1.68 mg/ml) in 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT with 0(□), 0.25 (○), 0.5 (●), 1 (▲), 3((□)), and 5 mM (Δ) PLP at 25°C. Aliquots were withdrawn at the indicated time intervals and assayed the residual activity as described in the "Experimental Procedure". The inset shows a plot of observed pseudo-first order rate constant *vs*. initial PLP concentrations, from which a second order rate constant was obtained.

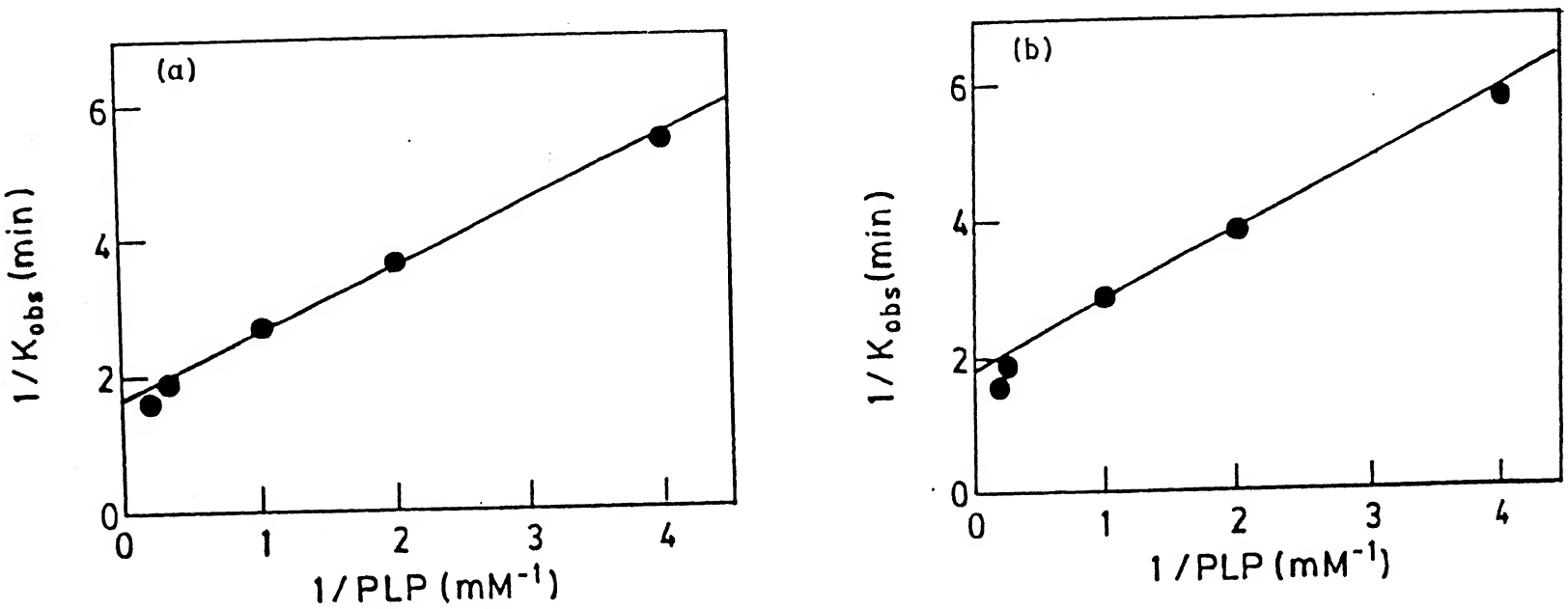


Figure III.6(a) & (b): **(a)** Double reciprocal plot of the pseudo-first order rate constants for inactivation of overall FAS against PLP concentration (data from Fig.4). **(b)** Double reciprocal plot of the pseudo-first order rate constants for inactivation of enoyl-CoA reductase against PLP concentration (data from fig.5)

the total enzyme concentration (e) is the sum of the concentrations of free enzyme and the complexes, as described by Chen and Engel [28].

$$e=E\{1+\frac{k_1}{k_2}(\frac{1+k_3}{k_4})[\text{pyridoxal-P}]\} \quad \text{----------eq(3)}$$

and fractional residual activity can be given by,

$$R=\frac{1+(k_1/k_2)[\text{pyridoxal-P}]}{1+(k_1/k_2).[\text{pyridoxal-P}].(1+k_3/k_4)} \quad \text{----------eq(4)}$$

Assuming that the covalent complex is totally inactive and the non-covalent complex dissociates rapidly on dilution into an assay mixture, a rearrangement of this equation gives,

$$1/[\text{pyridoxal-P}]=\frac{k_1 k_3}{k_2 k_4}\,1/(1-R)-(1+k_3/k_4)k_1/k_2 \quad \text{---------eq(5)}$$

Where R is the fractional residual activity.

Thus, a plot of 1/[pyridoxal-P] versus 1/(1-R) should be linear with an intercept on the 1/[pyridoxal-P] axis of

$-(1+k_3/k_4)(k_1/k_2)$ and a slope of (k_1k_3/k_2k_4).

The abscissa intercept represents $1+(k_4/k_3)$, the reciprocal of the maximum inactivation observed with an infinite concentration of PLP. Equilibrium analysis of the data obtained with subsaturating concentrations of PLP was carried out using

the (eq 5). The plot of 1/PLP versus 1/1-R yielded a straight line (Fig III.1&2, inset) and from the absicca intercept it was found that 5% of the activity was remaining at the saturating concentration of PLP. Non-linear regression analysis of (eq 4) gave k_1(0.955 min^{-1}) , k_2(1.31 min^{-1}), k_4(0.058 min^{-1}) and an another estimate of k_1(1.37 mM). The slight discrepancy observed in k_1 from kinetic and equilibrium analysis might be due to inherent differences in these two approaches. The calculated value of $k_2=(k_3/k_4)$ was 10.12 mM, showing that the equilibrium lies far towards the Schiff's base formation. The values obtained were almost identical for overall and enoyl-CoA reductase activity. Similar kinetic and equilibrium analysis on goose uropygial gland FAS have shown the higher value for the rate constants, dissociation constant and equilibrium constant suggesting that lysine residues are more susceptable towards the modification by PLP [34].

Inhibition of both the overall and enoyl-CoA reductase activities by PLP were readily reversible. On 80 fold dilution of the reaction mixture 95% of initial activity was recovered in 60 min (Fig III.7). Reduction with sodium borohydride after PLP treatment rendered the inactivation irreversible. According to the proposed model of Chen and Engel [38] the reversal should follow first order kinetics at least in the initial

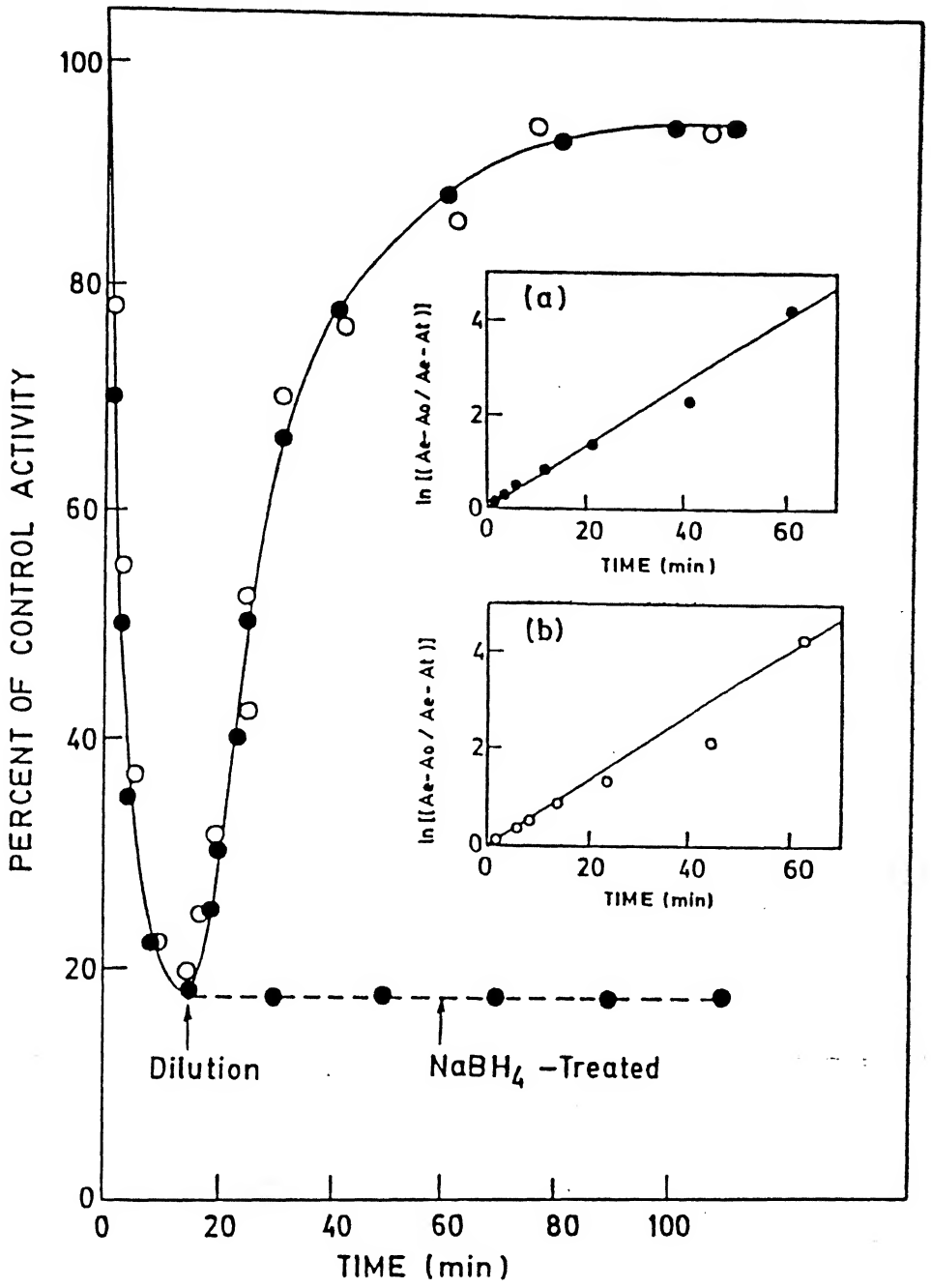


Figure III.7: *Time course of reversal of inhibition of overall FAS* (●) *and enoyl-CoA reductase* (o) *activities upon dilution of the PLP-treated FAS*. The enzyme (4 mg/ml) after incubation with 1 mM PLP at 25°C for 15 min was subjected to dilution (80-fold) or sodium borohydride (10 mM) treatment prior to dilution as described in "Experimantal Procedures". Inset: First-order plot of the reactivation of **(a):** overall FAS and **(b):** enoyl-CoA reductase by dilution of the PLP-treated enzyme. A_e and A_0 represent enzymic activity at the maximal level of reactivation and at zero-time following dilution, respectively. A_t, activity at any time t.

stage of reactivation, since the dissociation of noncovalent complex is rapid as compared to the reversal of the schiff's base. In fact this was found to be true (Fig III.7, inset) and estimation of k_4 from this analysis gave a value of 0.065 min^{-1} and calculation of the equilibrium constant k_3/k_4 using the value of k_3 obtained from kinetic analysis yielded a value of 9 mM . All these values are in agreement with those derived from equilibrium analysis. This value of k_4 can be combined with k_3 (obtained from kinetic analysis) to predict the residual activity remaining at the saturating concentration of PLP. It was calculated to be 9% which is reasonably close to the value obtained from the plot of 1/PLP versus 1/1-R as well as from non-linear regression analysis.

III.3.3 Protection experiments

In order to ascertain that the modification by pyridoxal 5'-phosphate occurs at the active site, protection experiments were performed using substrates and their analogs. Acetyl-CoA, malonyl-CoA and NADH did not provide any significant protection. The extent of protection with NADPH was increased with the increase of concentration and 5 mM NADPH provided 68% protection (Table III.1). Therfore it is apparent that the lysine residue modified is present at or near the NADPH binding

site.

Table III.1

The enzyme (1.68 mg/ml) was incubated with the ligands for 15 min prior to the addition of 1 mM PLP in dark. After 15 min of incubation overall FAS and enoyl-CoA reductase activities were measured immediately as described in section III.2.2.4.

PROTECTING LIGAND	PERCENT OF REMAINING ACTIVITY	
	FAS	ENOYL-CoA REDUCTASE
None	18	20
Acetyl-CoA (1 mM)	18	–
Malonyl-CoA (1 mM)	19	–
NADH (5 mM)	19	19
NADPH (1 mM)	74	81
NADPH (5 mM)	85	95

III.3.4 Spectral analysis of PLP modified FAS

It is known that the reaction of PLP with an ε-amino group of lysine results in the formation of a protonated Schiff's base which absorbs in the region of 410 to 435 nm [39,40]. The absorption spectrum of the fatty acid synthetase-PLP complex showed a maximum at 440 nm (Fig III.8), thereby indicating the formation of a protonated Schiff's base. This absorption peak disappeared on reduction with $NaBH_4$, and the reduced PLP-enzyme complex showed an absorption band at 323 nm which is characteristic of a reduced PLP-Schiff base [41]. The enzyme, treated with pyridoxal 5'-phosphate followed by reduction with sodium borohydride, exhibited a fluorescence emission spectrum with a maximum at 397 nm after uv irradiation at 325 nm and the fluorescence excitation spectrum (λ_e=397 nm) showed a maxima at 325 nm (Fig III.9). These results were consistent with the formation of N^{ε}-phosphopyridoxyllysine.

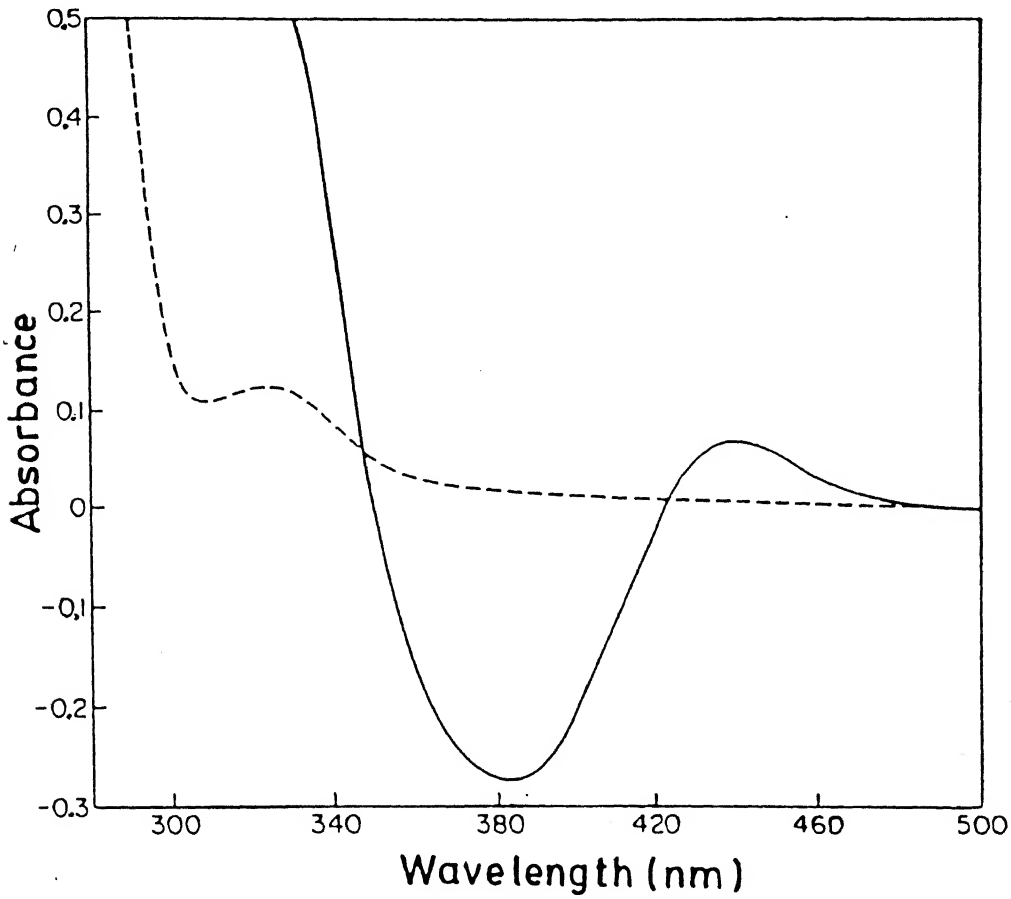


Figure III.8: *The absorption spectra of the fatty acid synthetase-PLP complex before and after reduction with sodium borohydride.* FAS (0.5 mg/ml) in 0.2 M potassium phosphate buffer,pH 7.0, containing 1mM EDTA and 1 mM dithiothreitol was treated with 1 mM PLP for 15 min The absorbance of this solution was determined against an equivalent concentration (1 mM) of PLP in the same buffer(——). For the spectrum of reduced enzyme-PLP complex, FAS complexed with PLP was treated with 10 mM sodium borohydride. For details, see "Experimental Procedure". The spectrum of the reduced complex (----) was determined against the same buffer.

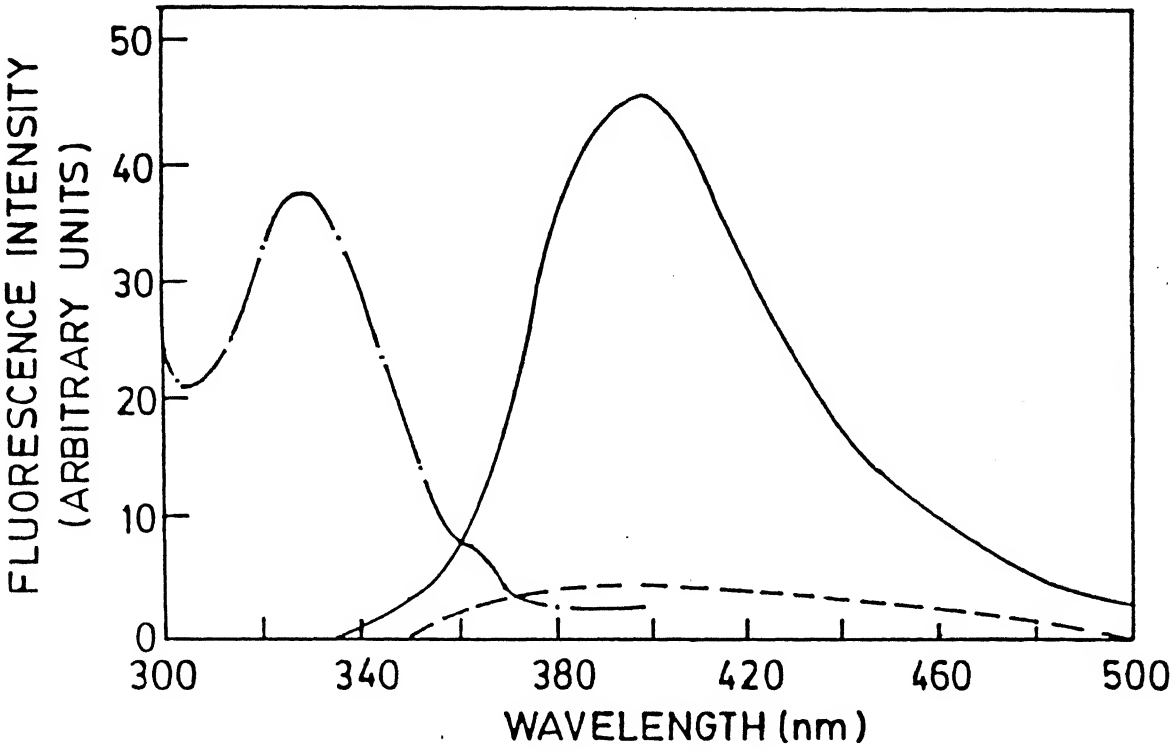


Figure III.9: *The fluorescence spectrum of FAS after reduction with sodium borohydride.* Fluorescence emission spectrum on excitation at 325 nn(———) and fluorescence excitation spectrum (λ_{em} =397 nm) (— - —) of the PLP-sodium borohydride reduced enzyme and the emission spectrum of pure enzyme (----) on excitation at 325 nm. FAS (0.5 mg/ml) was incubnated with 1 mM PLP under the reaction conditions for 15 min before reduction with sodium borohydride. For details, see "Experimental Procedures".

III.3.5 Stoichiometry of inactivation of FAS PLP

The stoichiometry of inactivation by PLP was determined by absorbance measurements at 325 nm after reduction with sodium borohydride. Since NADPH protected the enzyme against inactivation, lysine residues modified by PLP was determined in the absence and presence of NADPH. The number of nonessential lysine residues modified was determined by incubating the enzyme under identical conditions in the presence of NADPH. The number of lysine residues modified in the presence of NADPH was modified in the absence of NADPH to give the number of essential lysine residues modified (Table III.2). Results indicated that out of four modified lysine residues two were essential for FAS activity, similar to that found in uropygial gland of the goose [34]. This stoichiometry is different from the yeast FAS, where 40 lysine resedues were modified and six were essential [13]. The present evidence that two lysine residues per mole of pigeon liver FAS are essential for the enoyl-CoA reductase can be taken for the fact that there are two enoyl reductase sites per enzyme molecule .Such a conclusion is consistent with the hypothesis that animal FAS

consists of two identical peptides each containing the active sites for all of the steps involved in the fatty acid synthesis.

Table III.2

Pigeon liver FAS (2 mg/ml) was incubated with 1 mM of PLP. The Schiff's base was reduced with sodium borohydride and the concentration of N^{ε} phosohopyridoxyllysine was determined as described in section III.2.2.8.

CONDITION	PERCENT OF CONTROL ACTIVITY		MOL OF N^{ε} PHOSPHO PYRIDOXYLLYSINE PER MOL OF ENZYME[a]
	FAS	ENOYL-CoA REDUCTASE	
None	100	100	
Inactivated enzyme	18	20	4.35 ± 0.43
NADPH Protected enzyme	85	95	$2.25^{+} - 0.7$

[a]Number of determinations=5

III.3.6 Photoinactivation of FAS using pyridoxal 5'-phosphate as photosensitizer

Incubation of purified pigeon liver fatty acid synthetase with PLP in the dark and in the light resulted in inactivation

of overall FAS activity. A comparative study of the two samples, one kept in light and the other in dark, is shown in (Fig III.10). Similar kinetics of inactivation was obtained for the first 8 min in both the cases. However, while inactivation of the nonilluminated sample reached equilibrium at 18% of residual activity after 10-30 min of treatment, the inactivation of the illuminated sample leveled at 25% of residual activity in 20 min. The decrease in inactivation and leveling of the residual activity in illuminated sample could be explained by a light mediated destruction of PLP [18] under the experimental condition. NADPH showed a 60% of protection of the illuminated sample suggesting, modification of the essential residues are involved in the binding of the coenzyme. Although the PLP-treated enzyme in the dark was reactivated by subsequent dilution with the buffer but the illuminated enzyme sample was not reactivated on dilution (Fig.III.10). These results indicate that the photoinactivation of the FAS is an irreversible process. The extent of inactivation and reactivation of the enoyl-CoA reductase activity was exactly similar to the overall activity of FAS.

III.3.7 Spectral analysis of photoinactivated FAS

Spectral analysis of the pyridoxal-phosphate treated

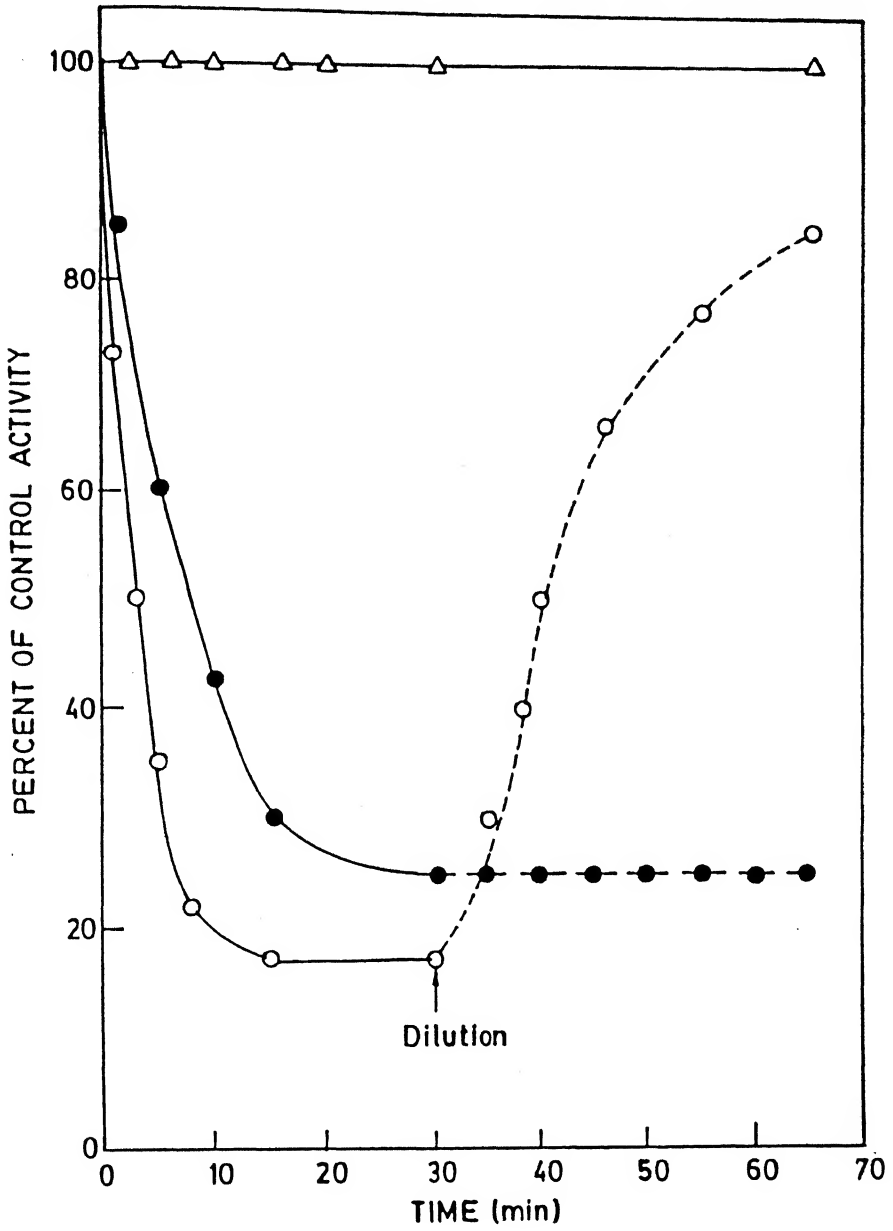


Figure III.10: *A comparative study of inactivation of illuminated and nonilluminated sample in the presence of PLP and reactivation upon dilution.* The enzyme (1.68 mg/ml) was incubated with 1 mM PLP under an oxygen atmosphere in the light (●) or in the dark (o). After 30 min (arrow) both the reaction mixture was diluted (80 fold) and simultaneously, the light was switched off and oxygen flow was stopped. At the indicated time intervals the aliquots were withdrawn and assayed for activity.

fatty acid synthetase in the light showed a fluorescence emission spectrum with a maxima at 445 nm after uv irradiation at 325 nm and the fluorescence excitation spectrum (λ_{em}=445 nm) showed a maxima at 325 nm (Fig III.11). Spectral properties of the enzyme modified in the dark did not change with respect to the samples that were illuminated or kept in the dark in the absence of PLP. This result suggested that the environment of PLP bound to the enzyme is altered by a nearby group resulting in appearance of a new fluorescence maxima at 445 nm when excited at 325 nm in the absence of reduction with sodium borohydride. The observation suggested that the Schiff's base formed between PLP and an active site lysine of FAS has undergone an attack by a nearby nucleophilic group resulting in an irreversible and inactive enzyme-PLP complex similar to the formation of X-azolidine-like structure (Fig III.12) as in the malate dehydrogenase enzyme [42]. This is considered from the known reactions of PLP with amino thiols [43] and various peptides and proteins [44]. Earlier specific chemical modification studies have shown that the lysine and histidine residues are essential for the enoyl-CoA reductase activity in chicken liver FAS [11]. Present investigations with pigeon liver FAS also showed that lysine and histidine residues are present in enoyl-CoA reductase domain (section III.3.10). It is

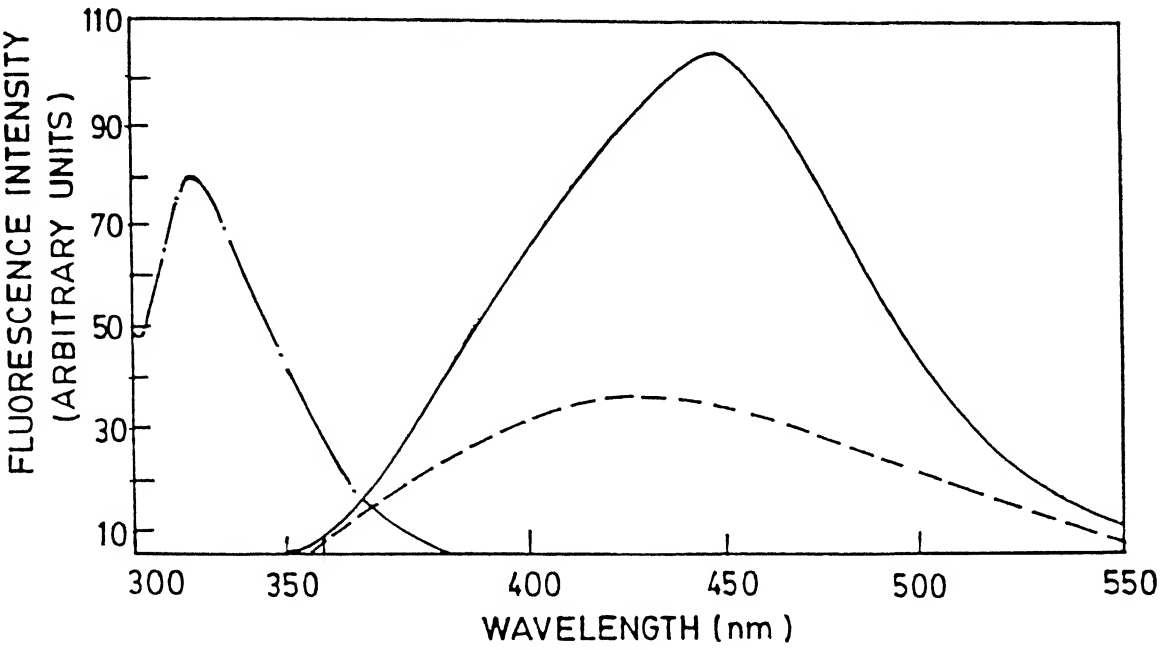


Figure III.11: *Fluorescence spectrum of the photoinactivated FAS.* Fluorescence emission spectrum on excitation at 325 nm (———) and fluorescence excitation spectrum (λ_{em} = 445 nm) (— - —) of the photoinactivated FAS and the emission spectrum of a similar sample (---) modified with PLP in dark on excitation at 325 nm. The FAS (2 mg/ml) was incubated with 1 mM PLP at 25°C under light and dark conditions (see marerials and methods) for 60 min. Then, the excess reagent was removed by filtering twice through Sephadex G-50 according to Penefsky before taking the spectra.

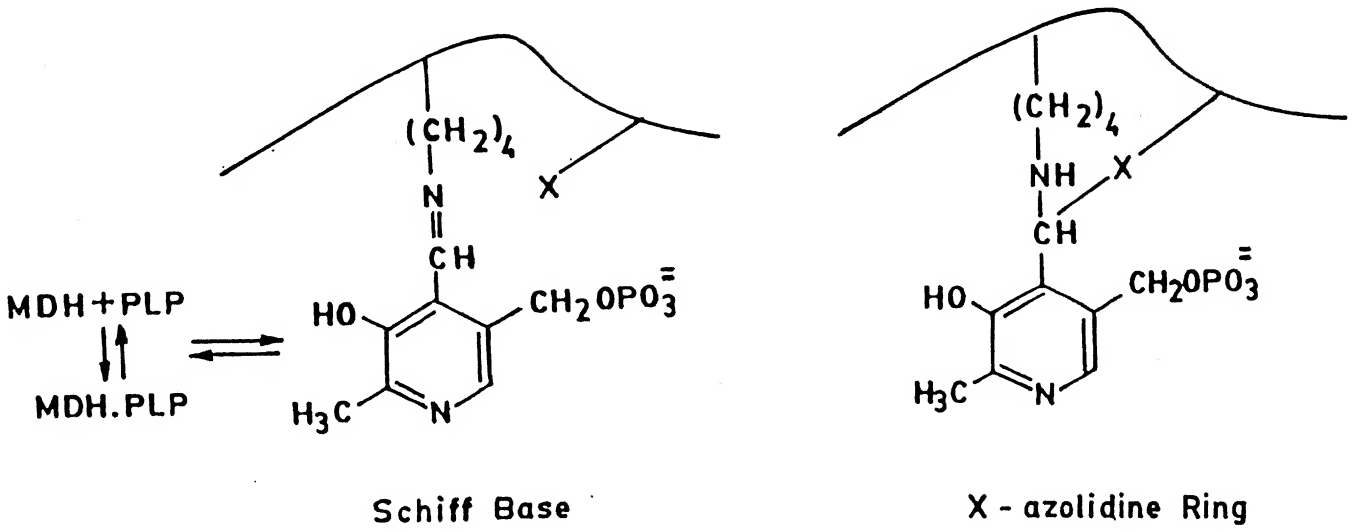


Figure III.12: Proposed mechanism of inactivation of malate dehydrogenase by pyridoxal 5'-phosphate (PLP).

reasonable to assume that imidazole groups may be the nucleophilic agent involved in the photomodification. However, exact nature of nucleophilic agent modified by photooxidation can only be known by amino acid analysis of the modified enzyme. This is the first report on the proximal presence of lysine and histidine residues in enoyl-CoA reductase domain of pigeon liver FAS.

III.3.8 Inactivation of FAS by 2,4,6-trinitrobenzenesulphonic acid

The time course of inactivation of FAS by TNBS is shown in (Fig III.13). The inactivation was biphasic since the reaction was carried out under pseudo-first order conditions. The biphasic curve consists one very rapid phase leading to 60% of inactivation in 2 min and one slow phase leading to 90% of inactivation in 30 min (Fig III.13, Inset) The inactivation by TNBS was irreversible as dialysis and sephadex G-25 gel filtration of the modified enzyme did not reverse its inhibition. TNBS at neutral pH can react with both thiol and amino groups. To determine the specificity of TNBS for amino groups of enzyme under the experimental conditions the thiol content of modified and unmodified enzyme was measured. There was no change in the number of thiol groups in the 90%

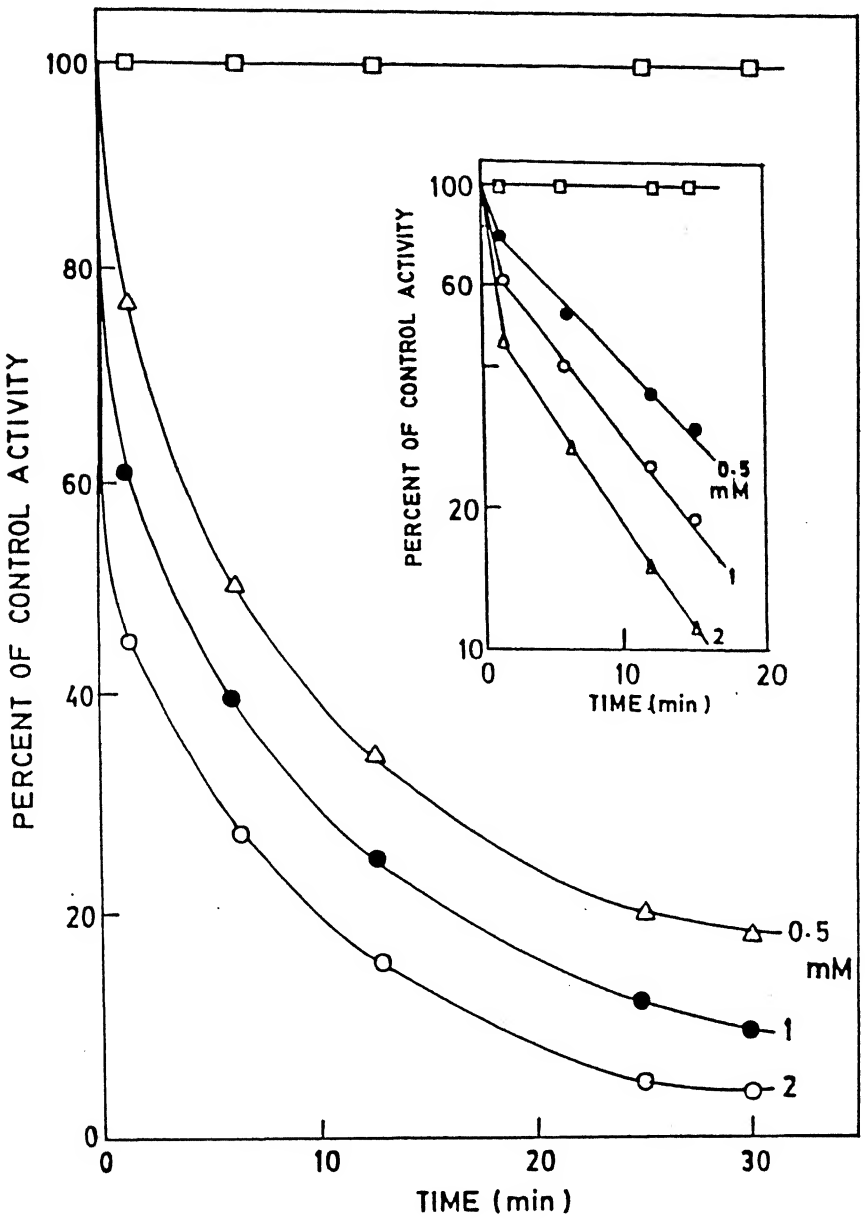


Figure III.13: *Inactivation of FAS by TNBS as a function of time* Enzyme (2 mg/ml) was incubated with variying concentration of TNBS as described under Materials and Methods. Millimolar modifying reagent concentrations used were 0.5 (Δ), 1 (●), and 2 (o). Inset: A semilogarithmic plot of residual activity *vs* time.

inactivated enzyme. The spectra of TNBS modified enzyme recorded in the range 250 to 500 nm (Fig III.14) was characteristic of the spectra of ε-TNP lysine complex as prepared and described earlier [16]. In the protection experiments acetyl-CoA, malonyl-CoA and NADH did not show any sigificant protection against inactivation by TNBS whereas, 5 mM NADPH showed 40% protection. This suggested that TNBS modified the lysine residue that is present at or near the NADPH binding site of the enoyl-CoA reductase domain of FAS.

III.3.9 Determination of number of lysine residues involved in reaction of FAS and TNBS

The stoichiometry of inhibition by TNBS was determined by the absorbance increase at 367 nm. The percentage of residual FAS activity and enoyl-CoA reductase activity was plotted as a function of number of residue modified (Fig III.15). Four lysine residues per molecule of enzyme were modified with 90% loss of activity. Extrapolation of the plot to zero enzyme activity showed that two moles of lysine residues per mole of enzyme were essential for the enzyme activity. Similar stoichiometry was also found in inactivation of the enzyme with PLP.

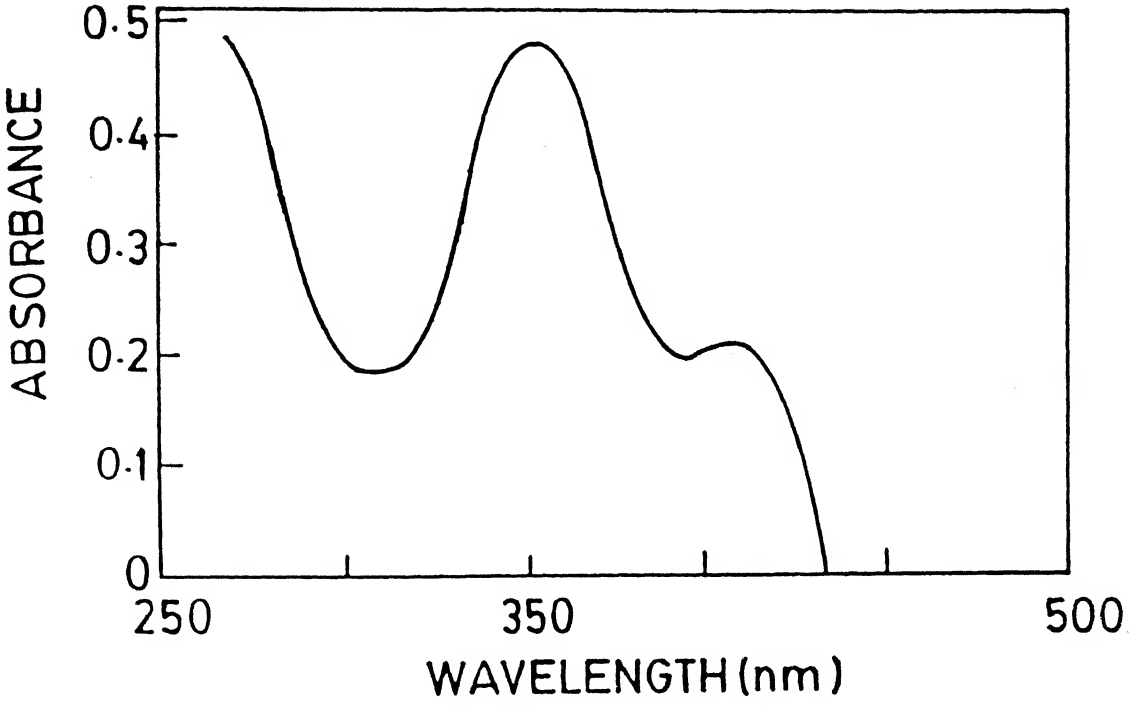


Figure III.14: *Absorbance spectra of ε-TNP-lysine of FAS.* The enzyme (0.5 mg/ml) was incubated for 30 min with 0.5 mM TNBS in 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. The absorbance of this solution was determined against a blank containing an equivalent concentration (0.5 mM) of TNBS in the same buffer.

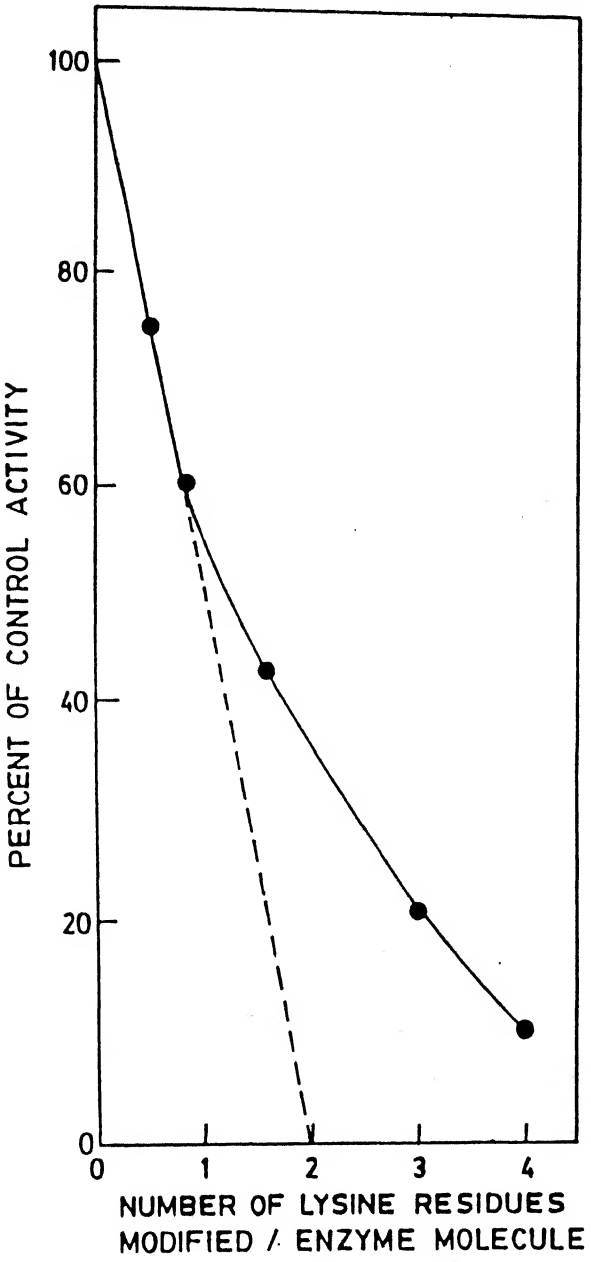


Figure III.15: *The stoichiometry of inactivation of FAS by TNBS*. The enzyme (4 mg/ml) was incubated with 1 mM TNBS under the reaction conditions as described in the "Experimental Procedure". The amount of trinitrophenyl derivative of ε- amino group of lysine residue formed at different time intervals during the enzyme inactivation was detremined by absorbance increase at 367 nm. In a parallel experiment the aliquots were withdrawn from the incubation mixture at same time intervals and the residual enzyme activity was detremined.

III.3.10 Modification of FAS by diethyl pyrocarbonate

Incubation of fatty acid synthetase with stoichiometric concentrations of diethyl pyrocarbonate resulted in the time dependent loss of synthetase and enoyl-CoA reductase activities. The inactivation follwed a typical bimolecular kinetic process as indicated by the plot of ln [(A-P)/(B-P)] versus time (Fig III.16 a&b) which were linear up to >90% of inactivation. The similar second order rate constants (k_2=11 $mM^{-1}min^{-1}$) for the inactivation of both the activites were obtained from slopes of $k_2(A-B)$. This result suggested that the loss of synthetase activity is the direct consequence of inability of the ethoxyformylated enzyme to catalyze the reduction of unsaturated acyl group i.e. the loss of enoyl-CoA reductase activity. In the above plot time was considered as $(1-e^{-K't}/K')$ where $K'=0.015$ min^{-1} is the first order rate constant for the decomposition of diethyl pyrocarbonate under the reaction conditions. This present result is different from the FAS of chicken liver, where the enzyme was inactivated by higher concentration of DEPC [11], but was quite similar to yeast alcohol dehydrogenase [45].

III.3.11 The effect of substrates and substrate analogs on the inactivation by diethylpyrocarbonate

In order to know the site of DEPC inhibition protection

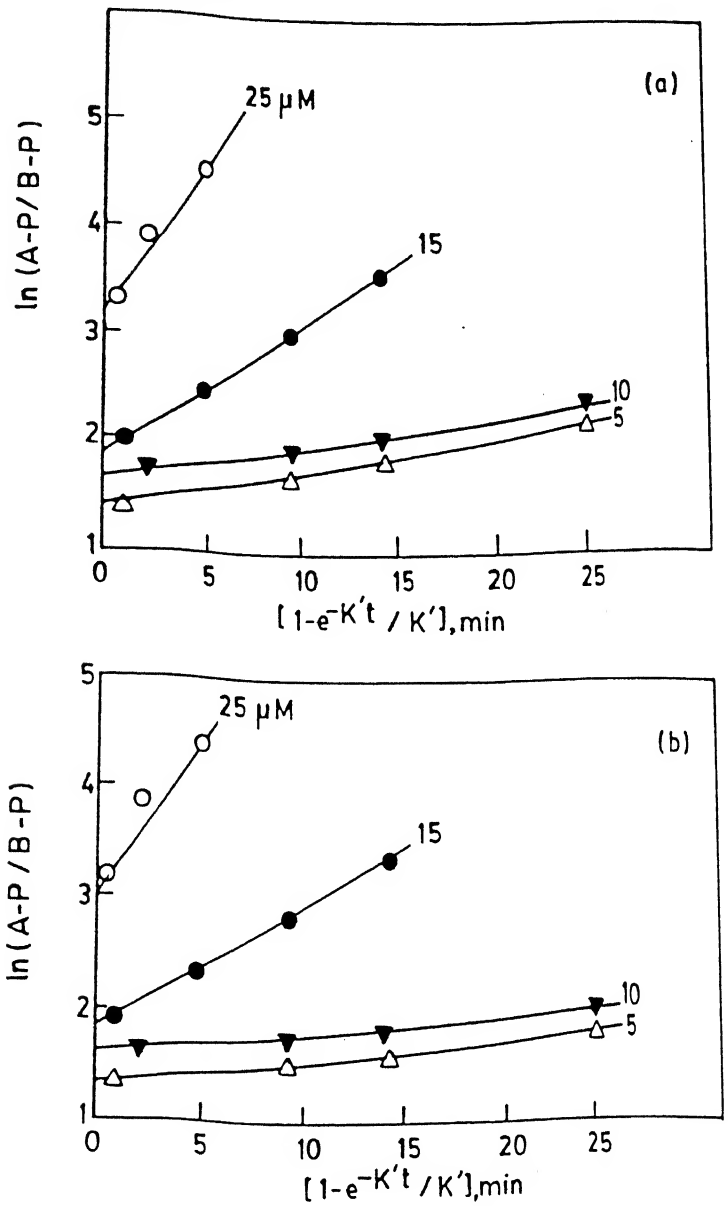


Figure III.16: *Time dependent inactivation of overall FAS* **(a)** *and enoyl-CoA reductase* **(b)** *activity by DEPC.* The enzyme (3 μM) in 0.2 M potassium phosphate buffer (pH 7.0), containing 1 mM EDTA and 1 mM DTT was incubated with 5 (Δ), 10 (▼), 15 (●), and 25 μM (O) DEPC at 0°C. Aliquots were were withdrawn at the indicated time intervals and the residual activity was determined as described in "Experimental Procedure".

studies were performed using substrates and their analogs. As shown in (Table III.3) acetyl-CoA and malonyl-CoA did not protect the enzyme from inactivation whereas, NADPH or NADP provided significant protection.

Table III.3

The enzyme (3 μM) was incubated with the protecting ligands for 10 min prior to the addition of diethyl pyrocarbonate (15 μM). After 15 min the overall FAS and enoyl-CoA reductase activities were determined.

LIGANDS	PERCENT OF REMAINING ACTIVITY	
	FAS	ENOYL-CoA REDUCTASE
None	5	6
Acetyl-CoA(1 mM)	6	5
Malonyl-CoA(1 mM)	5	-
NAD^+(5 mM)	5	5
5'-AMP(5 mM)	6	5
2'-AMP(5 mM)	54	61
2',5'-ADP(5 mM)	40	63
NADPH(5 mM)	50	68
$NADP^+$(5 mM)	46	64

Similar extent of protection was afforded by 2′-AMP or 2′-5′ADP but not by NAD, NADH or 5′-AMP which indicated that 2′-phosphate group interacts with the reactive histidine residue in the binding process.

III.3.12 Characterization of the product formed by the reaction of diethylpyrocarbonate with FAS

Diethyl pyrocarbonate has been used extensively as a histidine reagent to elucidate the role of this amino acid in a number of enzymes [33,46-49]. Although diethyl pyrocarbonate exhibits specificity towards histidine residues [50,51], it is also reactive with tyrosine, cysteine, lysine and arginine under certain conditions [53] Fatty acid synthetase (3 μM) when incubated with diethyl pyrocarbonate (15 μM) for 15 min, resulted in complete loss of the enzyme activity. The product formed was analyzed by absorption spectroscopy.

The modified enzyme showed an absorption band at 240 nm characteristic of ethoxyformylhistidine [52] and the involvement of essential tyrosine residue was ruled out, since the absorption at 278 nm [53] was unchanged by ethoxyformylation, both shown in (Fig.III.17). The possibility of ethoxyformylation of essential cysteine residues was examined in an experiment of measurement of thiol content as

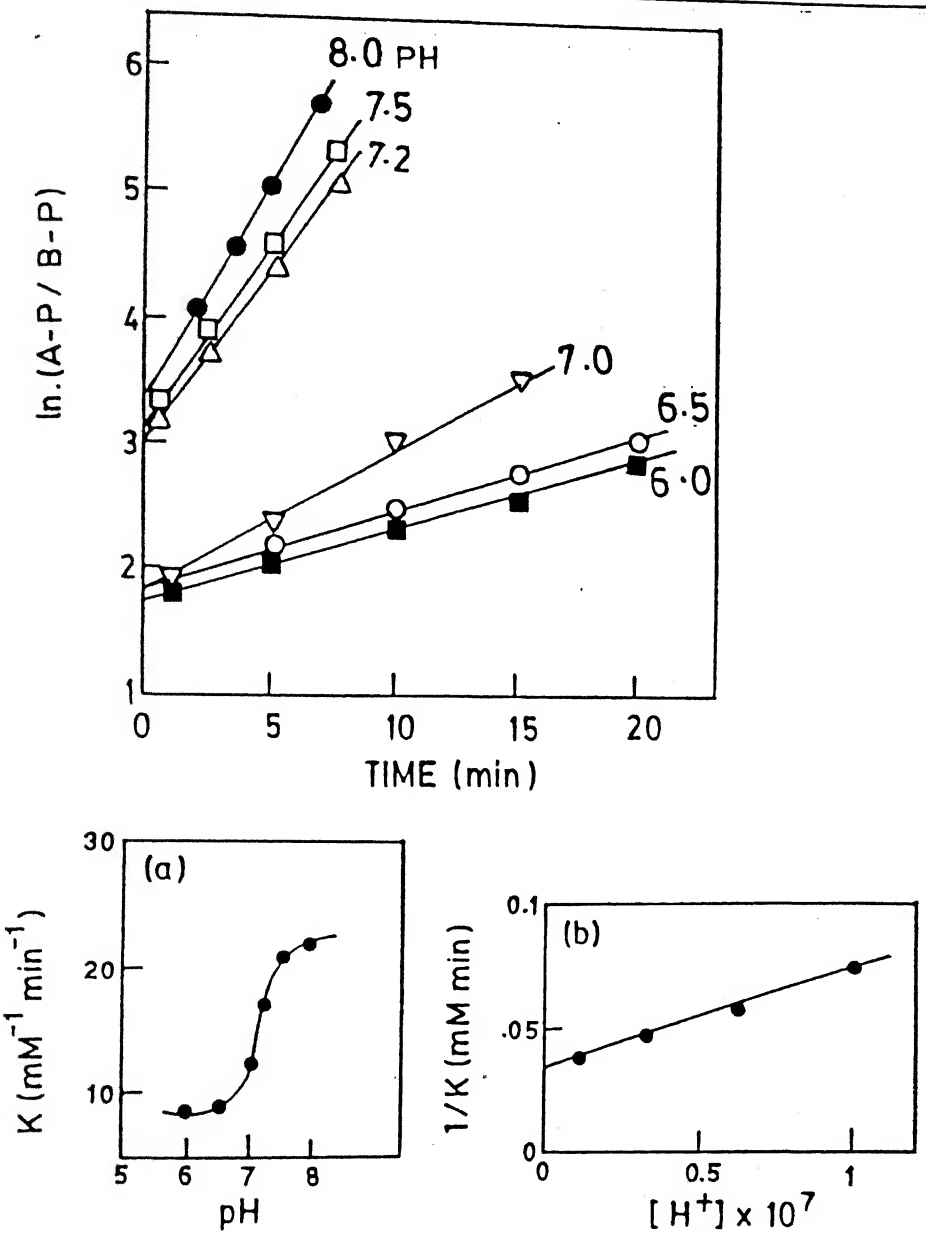


Figure III.17: *Fatty acid synthetase inactivation by DEPC as a function of pH.* The enzyme (3 μM) was inactivated with 6 μM of DEPC in 0.2 M potassium phosphate buffer over a pH range of 6.0-8.0. At indicated time the aliquots were removed and enzyme activity was determined. Inset **(a)**: Plot of dependence of second order rate constant of inactivation on pH, the second order rate constant K was determined as described in "Experimental Procedure" Inset **(b)**:1/K mM min *vs* $[H]^+$[reploting the data of **(a)**].

described in section III.2.2.11 using 3 μM of enzyme and 15 μM of DEPC. The thiol content measured was same in the modified and unmodified enzyme. The reversal of inhibition using hydroxylamine to almost 90% of original activity indicated the involvement of histidine residue in the DEPC reaction. Furthermore, studies on the pH-dependence of inactivation yielded a value of pK_a 7.0 consistent with that for histidine as shown in (Fig III.18).

III.3.13 Stoichiometry of the reaction of FAS with diethyl pyrocarbonate

The number of reactive histidine residues was determined by monitoring activity loss as a function of ethoxyformylation at 240 nm as shown in (Fig III.19). A linear plot was obtained which showed that two histidine residues reacted and resulted in the complete loss of the activity. These results concluded that diethyl pyrocarbonate modified one histidine residue at each enoyl-CoA reductase domain and this residue is located at the pyridine nucleotide binding site. Similar stoichiometry was found in FAS of chicken liver [11].

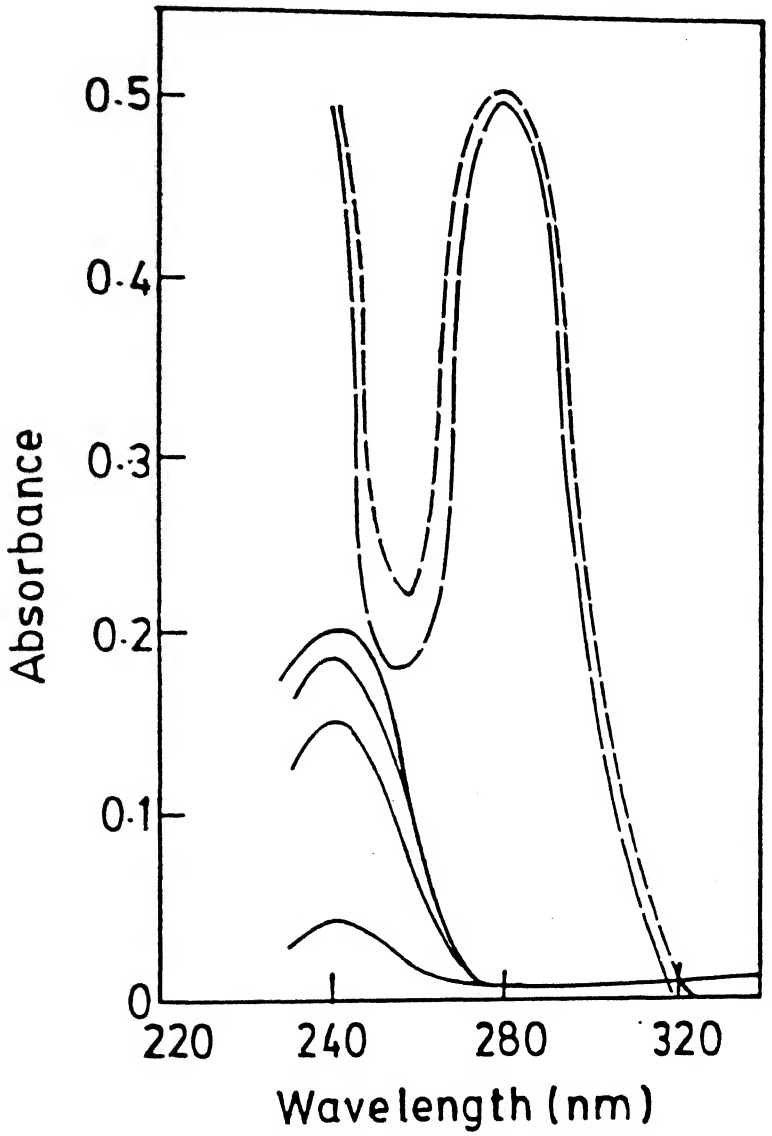


Figure III.18: *Effects of DEPC on the absorption spectra of FAS* FAS (3 μM) was incubated with 6 μM DEP and the absorption spectra were recorded at time intervals; 1, 5, 10, 15 min (———). Spectra were also recorded before (– – –) and after (—— —— ——) the treatment of FAS with 6 μM DEP for 15 min using a buffer blank

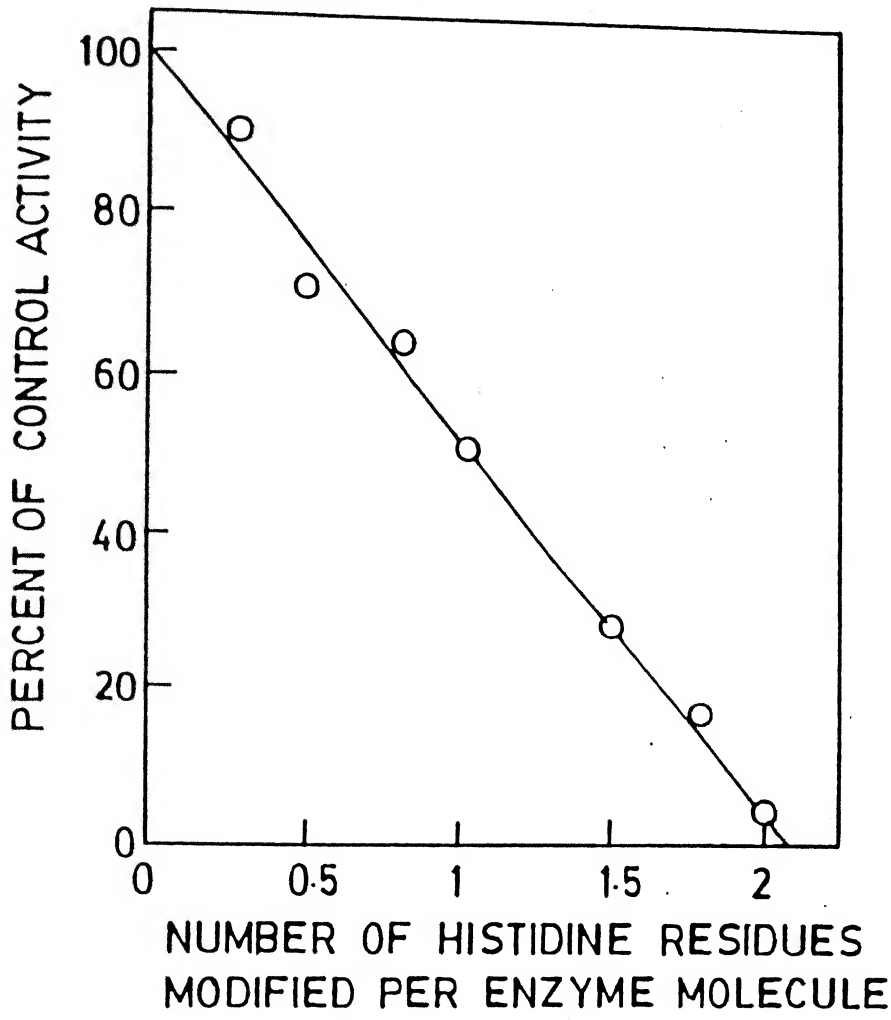


Figure III.19: *Stoichiometry of inactivation of FAS by DEPC.* The enzyme (3 μM) was incubated with 16 μM of DEP under the reaction conditions as described in the "Experimental Procedure". The concentration of ethoxyformyl histidine was determined by absorbance at 240 nm using an extinction coefficient of $3.2 \times 10^{3} cm^{-1} M^{-1}$. In a parallel experiment the aliquots were withdrawn from the incubation mixture at different time intervals and diluted in 50 mM neutralized N-acetylhistidine to terminate the reaction and assayed for synthetase activity.

III.4 SUMMARY

Pigeon liver fatty acid synthetase incubated with lysine specific reagents, PLP and TNBS resulted in the loss of overall and enoyl-CoA reductase activities. The inactivation was found dependent on concentration of the inhibitor and time. The inactivation of enzyme by TNBS was irreversible but PLP caused reversible inactivation as it could be reversed by dilution or dialysis. The loss of enzyme activity by both these inhibitors was prevented by NADPH and not by any of the other substrate. Modification of FAS in presence of light under an oxygen atmosphere resulted in an irreversible inactivation, which was protected by NADPH. Spectral analysis of the photomodified enzyme showed a fluorescence spectrum with a maxima at 445 nm after uv-irradiation at 325 nm, suggesting light mediated addition of a nucleophilic residue (probably an imidazole group) to the PLP-lysine azomethine bond. The modification of FAS enzyme with PLP is a two step reaction following a pseudo-first order kinetics, first one a rapid step to form noncovalent enzyme-PLP complex leading to a slow step for the formation of a covalent enzyme-PLP complex, a Schiff's base. The inactivation by TNBS was biphasic and followed pseudo-first order kinetics. Absorption and fluorescence studies clearly indicated the modification of lysine residue by

these two inhibitors. Stoichiometric results established that four lysine residues were modified per enzyme molecule by these inhibitors and two of them were essential. The present investigations have convincingly demonstrated the presence of one essential lysine residue per subunit at or near the NADPH binding site of the enoyl-CoA reductase domain of FAS

Pigeon liver fatty acid synthetase was also inactivated by the stoichiometric concentrations of histidine reagent, diethyl pyrocarbonate. Among two reductase activities DEPC inactivated the enoyl-CoA reductase activity. The inactivation followed a typical bimolecular process with the second order rate constant of 11 $mM^{-1}min^{-1}$. The inactivation of enzyme by DEPC was protected by NADPH, indicating that the modification occurs at the coenzyme binding site.

The specificity of diethyl pyrocarbonate for histidine residues was confirmed by the appearance of absorption band at 240 nm, reversal of enzyme activity by hydroxylamine treatment and no change in thiol content of the DEPC treated enzyme. Furthermore, studies on the pH-dependence of inactivation yielded a value for pK_a of 7.0 consistent with that for histidine. The stoichiometric result indicated that two histidines were modified upon complete inactivation of the fatty acid synthetase. These results also suggested that each

subunit of FAS contains the enoyl-CoA reductase site having one histidine residue at each enoyl reductase domain and is located at the pyridine nucleotide binding site. This is in agreement with the previous findings that each peptide contains a covalently attached phosphopantetheine and a thioesterase domain to support the homodimer model of this enzyme.

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CHAPTER IV

EVIDENCE FOR ARGININE RESIDUES PRESENT AT β-KETOACYL REDUCTASE AND ENOYL-CoA REDUCTASE DOMAINS OF PIGEON LIVER FATTY ACID SYNTHETASE

IV.1 INTRODUCTION

Pigeon liver fatty acid synthetase is a multifunctional enzyme complex which catalyses the synthesis of palmitic acid by seven different sequential enzymatic reactions [1-4]. Among these are the two NADPH dependent reduction reactions. First reduction is catalysed by β-ketoacyl reductase:

$$CH_3COCH_2COS\text{-}ACP + NADPH + H^+ \longleftrightarrow D\text{-}CH_3CHOHCH_2COS\text{-}ACP + NADP^+$$

whereas the second reduction reaction is catalysed by enoyl-CoA reductase

$$CH_3CH{=}CHCOS\text{-}ACP + NADPH + H^+ \longleftrightarrow CH_3CH_2CH_2COS\text{-}ACP + NADP^+$$

These two reductases exhibit high specificity for NADPH over NADH for all FAS, regardless of its source. Since arginine is

known to be involved in the binding of anionic substrates to several enzymes [5], it is possible that arginine residues may be involved in the binding of NADPH to FAS. Presence of arginine was investigated by two arginine specific reagents on pigeon liver FAS activity. Phenylglyoxal preferentially modifies arginine residues in different enzyme proteins [6]. The inactivation of pigeon liver FAS by phenylglyoxal was dependent on time and concentration of the reagent. Earlier it was reported that phenylglyoxal and 2,3-butanedione inactivates FAS of uropygial gland of goose and chicken liver by inhibiting the β-ketoacyl and enoyl reductases [7,8]. In Chapter III the presence of essential lysine and histidine residues has been demonstrated at the enoyl-CoA reductase domain only and not at the β-ketoacyl reductase of pigeon liver FAS. The present study has shown the presence of arginine residues at the active sites of β-ketoacyl reductase and enoyl-CoA reductase of pigeon liver FAS using phenylglyoxal and 2,3-butanedione.

IV.2 EXPERIMENTAL PROCEDURE

IV.2.1 MATERIALS

Acetyl-CoA, Malonyl-CoA, NADPH, Phenylglyoxal, 2,3-butanedione, 5,5′-dithiobis (2-nitrobenzoic acid), 2′-AMP, 2′-ADP, 5′-AMP and buffer components (Sigma), Sephadex G-25

(Pharmacia), Dithiothreitol (Calbiochem) were procured from the above designated sources. All other chemicals were of highest purity grade commercially available. Distilled water from Millipore Milli Q system was used in all the experiments. S-crotonyl-N-acetyl cysteamine and S-acetoacetyl-N-acetyl cysteamine were synthesized by the method as described in Chapter II, section 2.8

IV.2.2 METHODS

IV.2.2.1 Enzyme preparation and activity measurements

Pigeon liver fatty acid synthetase was purified and assayed spectrophotometrically by the method of Muesing and Porter [9] which is described in Chapter II. The enzyme was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the standard method of Laemmli [10] that exhibited a single band. Assays for β-ketoacyl and enoyl-CoA reductase activities were carried out spectrophotometrically as described earlier [11,12] in the Chapter-II, section 2.9. Fatty acid synthetase activity was measured by the decrease in the absorbance of NADPH at 340 nm on UV-vis spectrophotometer (Shimadzu, Model UV-160A). All measurements were carried out at 25°C.

IV.2.2.2 Calculation of specific activity of the enzyne

The purified enzyme used in all experiments had the specific activity of 35 U/mg of protein. One unit of the enzyme is defined as the amount of enzyme that will form one nmole of palmitate per minute. Calculations for enzyme unit has been described in chapter II, section 2.7.

IV.2.2.3 Inactivation studies of FAS with phenylglyoxal

The stock solution of phenylglyoxal was prepared in 1% ethanol. Solutions of NADPH and phenylglyoxal were prepared fresh daily and concentrations were determined spectrophotometrically using extinction coefficients of 6.22×10^3 $cm^{-1}M^{-1}$ at 340 nm, and $12.4 \times 10^3 cm^{-1}M^{-1}$ at 253 nm, respectively. Inactivation of FAS were carried out at 25°C in 0.2 M potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM DTT and 80 mM NH_4HCO_3 at a protein concentration of (3-12 μM) with various concentrations (1-7 mM) of phenylglyoxal. The incubations were performed in the dark to minimize light-induced decomposition of reagent [13]. At different time intervals, aliquots were withdrawn and assayed for overall FAS, enoyl-CoA and β-ketoacyl reductase activity. Controls without phenylglyoxal were run concurrently.

IV.2.2.4 Effect of substrates and substrate analogs on inactivation of FAS by phenylglyoxal

The enzyme (12 μM) was incubated with indicated concentration of substrates for 15 min prior to the addition of 2 mM phenylglyoxal. After 30 min aliquots were taken and assayed for overall and reductases activity.

IV.2.2.5 Modification of FAS with 2,3-butanedione

Modification of FAS at the concentration of (3-12 μM) with (10-40 mM) 2,3-butanedione was carried out in 20 mM borate buffer containing 200 mM KCl, 1 mM DTT and 1 mM EDTA at pH 7.5 and 25^{o}C. At different time intervals, aliquots were withdrawn and assayed for overall FAS and two reductase activities. Controls were run in absence of 2,3-butanedione concurrently.

IV.2.2.6 Effect of substrates and substrate analogs on inactivation of FAS by 2,3-butanedione

The enzyme (12 μM) was incubated with indicated concentration of substrates for 30 min prior to the addition of 30 mM 2,3 butanedione. After 30 min of incubation aliquots were taken and assayed for overall FAS and reductases activities.

IV.2.2.7 pH dependence studies of inactivation of FAS by phenylglyoxal

Effect of pH on inactivation profile was observed over a pH range of (6.5-8.0) using 0.2 M potasium phosphate buffer. Enzyme (2 mg/ml) was incubated with 7 mM phenylglyoxal in 0.2 M potassium phosphate buffer containing 1 mM EDTA and 1 mM DTT. Appropriate control measurements were also made under similar pH conditions. The effect of pH on enzyme inactivation was analyzed according to the equation-1 [14]

$$\frac{1}{k_{obs}} = \left[\frac{1}{k_{max}} . K\right] [H^+] + \frac{1}{k_{max}} \qquad (1)$$

Where k_{obs} is the pseudo-first order rate constant at each pH, k_{max} is the maximal pseudo-first order rate constant, K is the ionization constant of the group involved in the catalysis and $[H^+]$ is the hydrogen ion concentration.

IV.2.2.8 The stoichiometry of binding of phenylglyoxal with FAS

Fatty acid synthetase (4 mg/ml) was incubated with 2.5 mM phenylglyoxal for different time intervals. After incubation for the indicated time intervals the synthetase activity was determined and the reaction was terminated with 10 mM arginine. Excess reagent was removed by column centrifugation method

according to penefsky [15] as described in the chapter III and the absorbance was measured at 250 nm. Number of arginine residues modified were calculated by taking the ratio of concentration of diphenylglyoxal adduct and the enzyme. The concentration of diphenylglyoxal adduct and the enzyme were determined by using the absorbance coefficient of 11000 $cm^{-1}M^{-1}$ at 250 nm [16] and Lowry et. al method [17], respectively. Molecular weight of FAS was taken as 450,000. In another studies at the same time intervals aliquots were removed and assayed for reductases activities.

IV.3 Results and discussion

IV.3.1 Inactivation of FAS by phenylglyoxal

Incubation of the enzyme with phenylglyoxal resulted in time dependent loss of synthetase, β-ketoacyl reductase and enoyl-CoA reductase activities. The inactivation was found irreversible. Bicarbonate was chosen as the buffering ion in these experiments because of the specific enhancement of reaction rate. This enhancement is believed to be the result of complex formation between bicarbonate and the guanidinium group of arginine that lowers the pK_a and promotes nucleophilic attack by this group on the carbonyl carbon of phenylglyoxal

[13]. The rate of inactivation was dependent on the phenylglyoxal concentration. The rate of enzyme inactivation increased as the phenylglyoxal concentration increased and almost complete inactivation was observed at 7 mM concentration in 20 min (Fig. IV.1). The inactivation followed pseudo-first order kinetics, as shown by semi-log plots of residual activity versus time for overall FAS, enoyl-CoA reductase and β-ketoacyl reductase activity respectively (Fig. IV.2,3&4). The present result were in contrast to the inactivation of synthetase from goose uropygial gland by phenylglyoxal as shown by Poulose and Kolattukudy [7] that yielded more complex kinetic behavior and was similar to the chicken liver FAS [8]. Second order rate constants (k_2) for overall FAS, enoyl and β-ketoacyl reductase were obtained from slopes of linear plots of pseudo-first order rate constant (k_1) *vs* reagent concentration were $2.4x10^{-2}$, $1.1x10^{-2}$ and $1.3x10^{-2}$ $mM^{-1}min^{-1}$ respectively (Fig IV.2a) and (Fig IV.3 & 4, inset a), which is lower than the value obtained from chicken liver FAS [8]. The order of reaction (n) with respect to phenylglyoxal was determined from the slope of a plot according to the equation

$$\log k_1 = \log k_2 + n \log [R]$$

where, [R] represents reagent concentration.

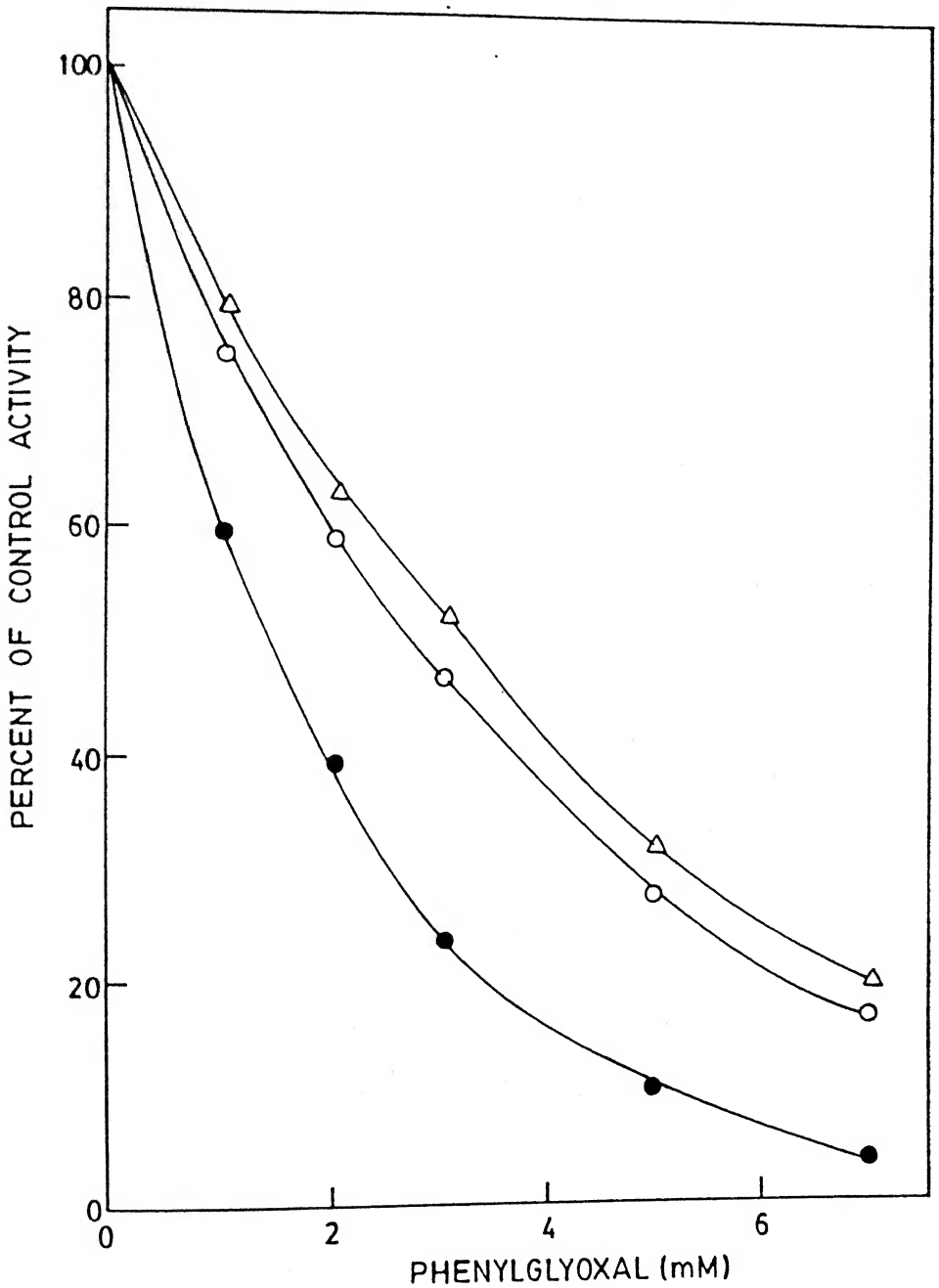

Figure IV.1: *Effect of phenylglyoxal concentrations on the inactivation of overall FAS, enoyl-CoA reductase and β-ketoacyl reductase activity.* Incubation with phenylglyoxal was carried out at a protein concentration of 3, 8, and 12 μM for FAS (●), β-ketoacyl reductase (o) and enoyl-CoA reductase (Δ) respectively with varying concentrations of phenylglyoxal (1-7 mM). The reaction time was 20 min and the assays for enzyme activity were carried out as described in "Experimental Procedure".

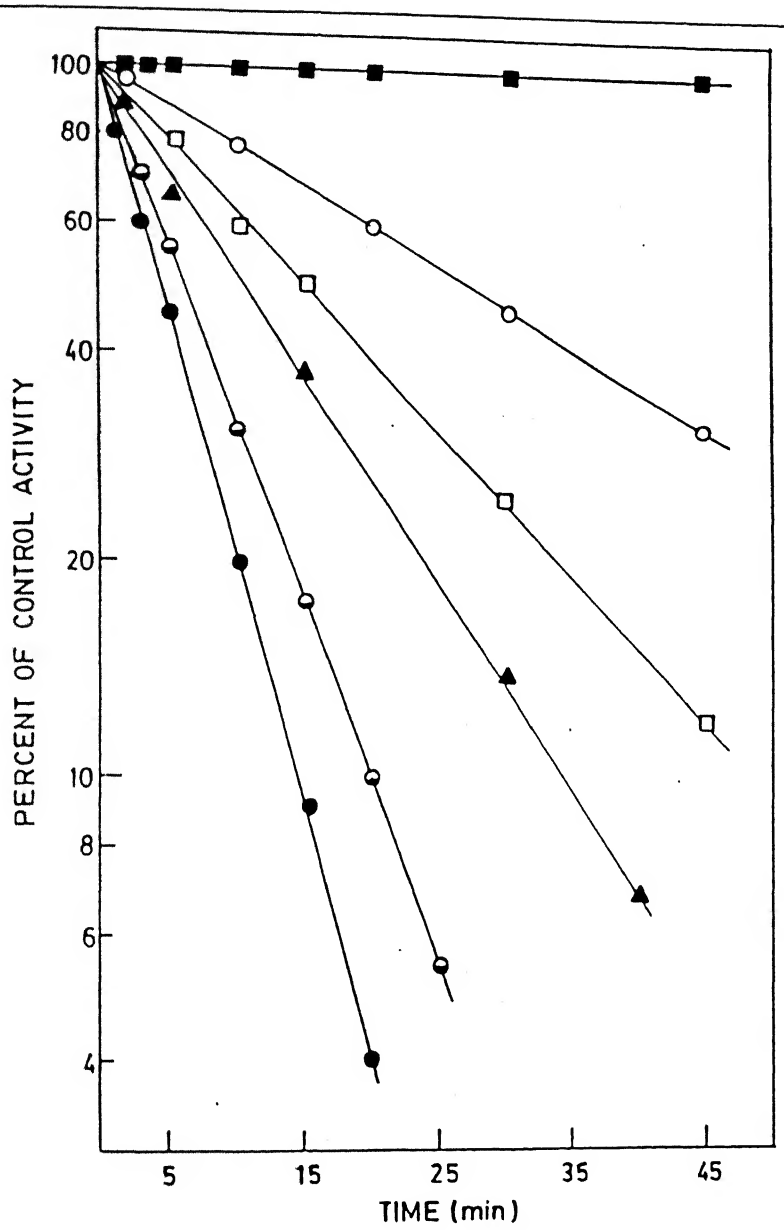

Figure IV.2: *Kinetics of the inactivation of overall FAS activity by phenylglyoxal* The enzyme (3 μM) in 0.2 M potassium phosphate buffer (pH 7.0), containing 1 mM EDTA and 1 mM DTT and 80 mM NH_4HCO_3 was incubated with 0(■), 1(○), 2(□), 3(▲), 5(⊖), and 7(●) mM phenylglyoxal at 25°C. Aliquots were withdrawn at the indicated time intervals and assayed the residual activity as described in the "Experimental Procedure".

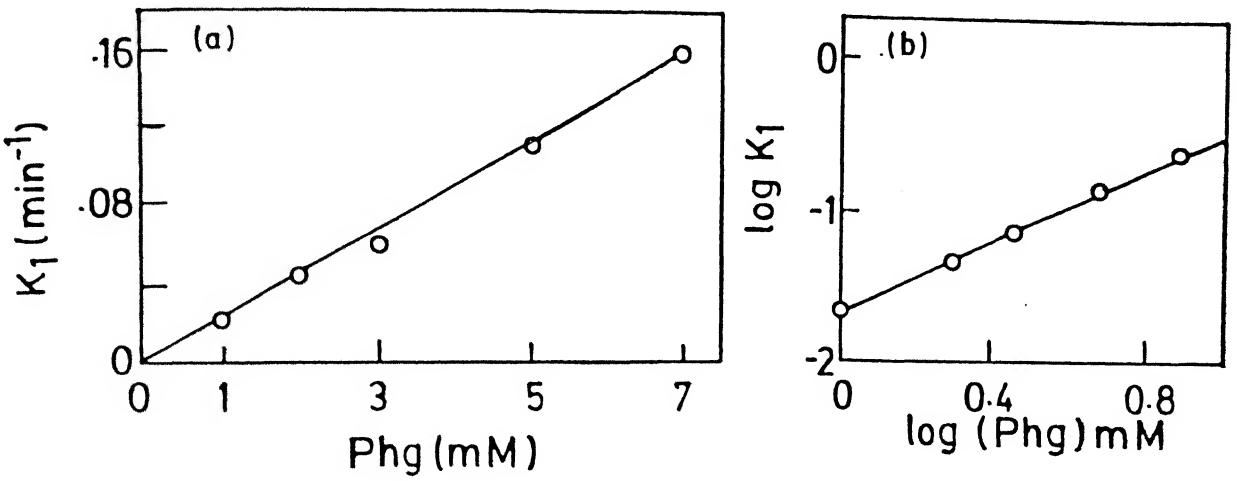

Figure IV.2(a) & (b): **(a) and (b)** are linear primary and double log plots of observed pseudo-first order rate constant *vs* phenylglyoxal concentrations.

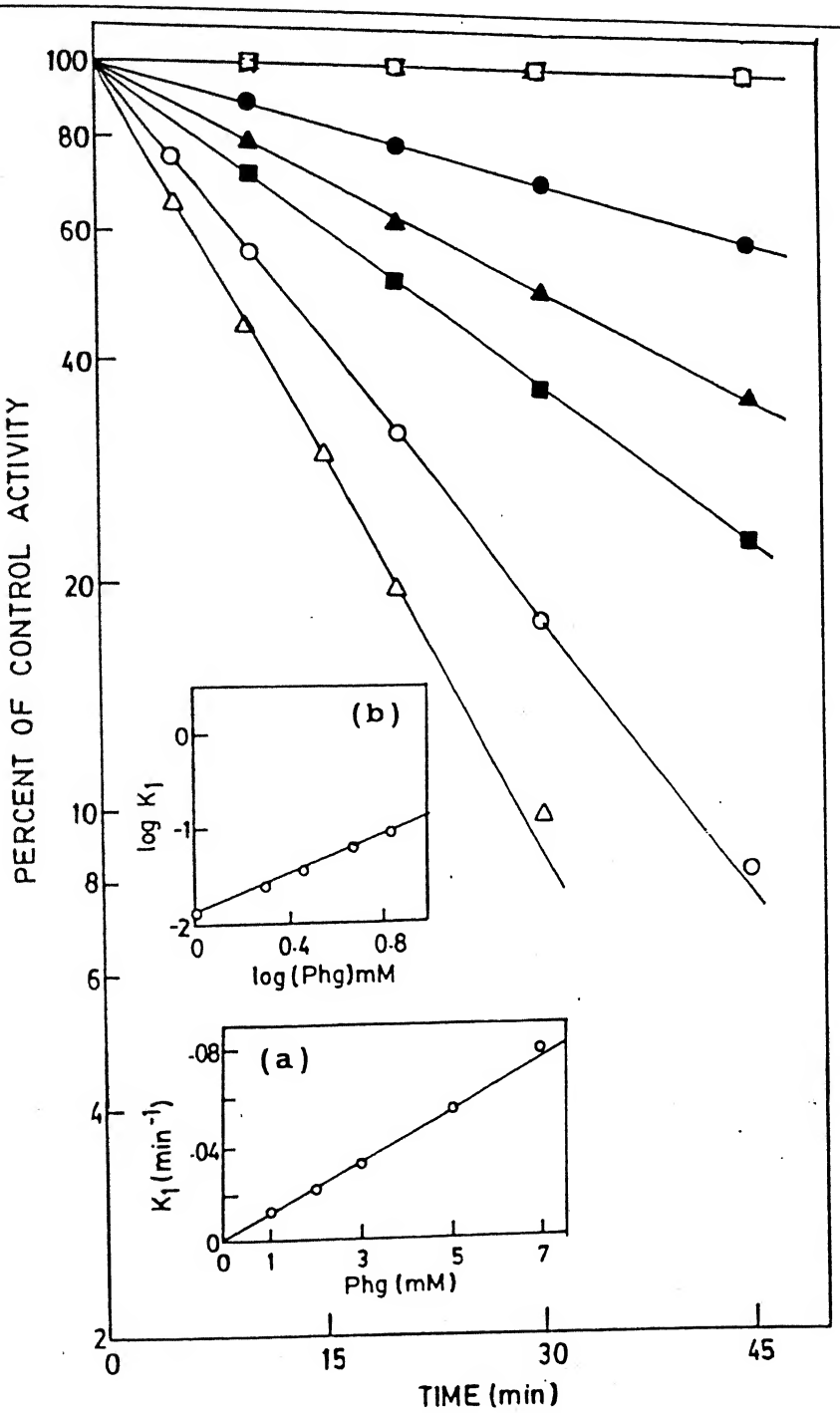

Figure IV.3: *Kinetics of the inactivation of enoyl-CoA reductase activity by phenylglyoxal*. The enzyme (12 μM) in 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT and 80 mM NH_4HCO_3 was incubated with 0 (□), 1(●), 2(▲), 3(■), 5(o), and 7(Δ) mM phenylglyoxal at 25°C. Aliquots were withdrawn at the indicated time intervals and assayed the residual activity as described in the "Experimental Procedure". Inset **(a)** and **(b)** are linear primary and double log plots of observed pseudo-first order rate constant *vs* phenylglyoxal concentrations.

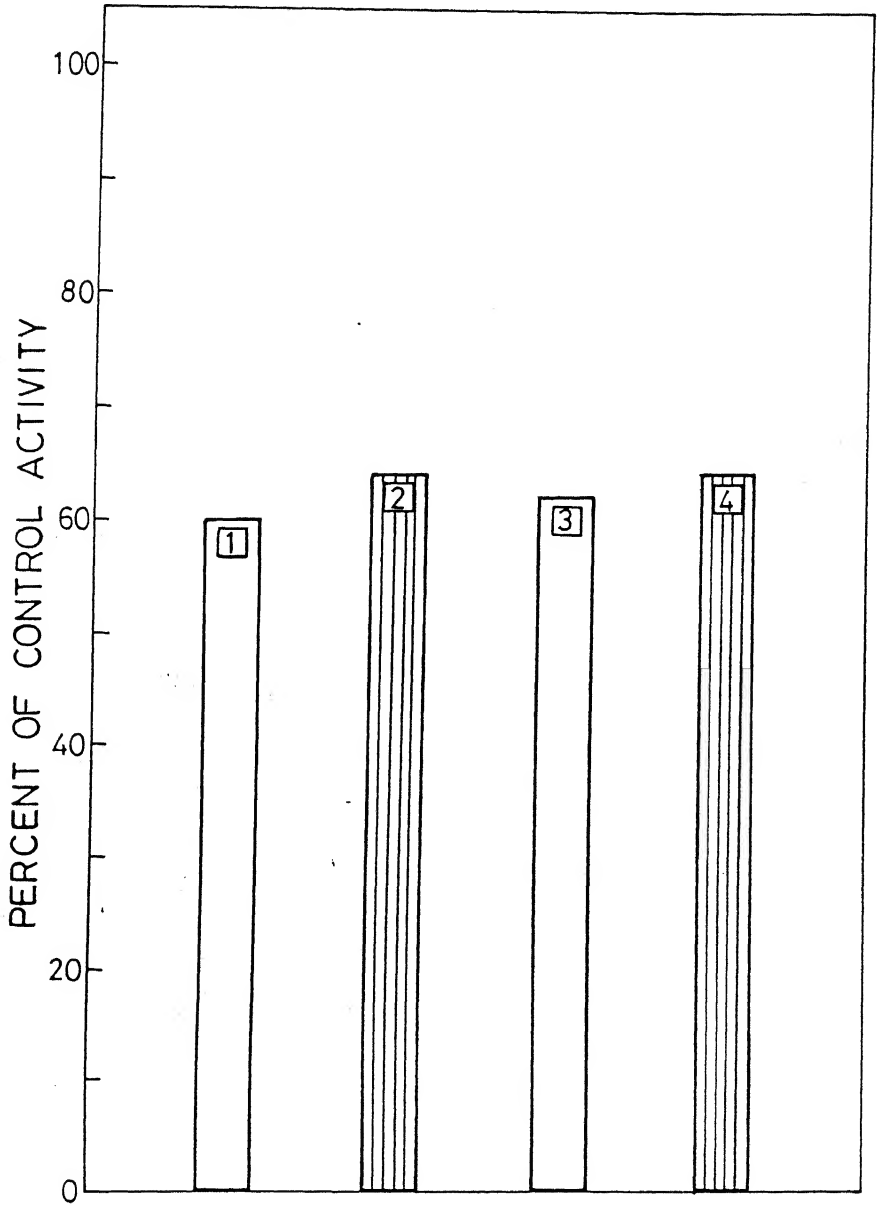

Figure IV.11: *Effect of L-arginine and NADH separately on the inactivation of octopine dehydrogenase by PCMS and o-phthalaldehyde.* Octopine dehydrogenase (0.152 mg/ml) was incubated with 5 μM PCMS for 21 min (bar 1). L-arginine (24 mM) treated enzyme was incubated with 20 μM o-phthalaldehyde followed by 5 μM PCMS for 25 and 21 min respectively (bar 2). In another set enzyme was incubated with 20 μM o-phthalaldehyde (bar 3) or with NADH (0.6 mM) prior to the addition of the above inhibitors for the same time period (bar 4). The residual enzyme activity was determined after indicated treatments.

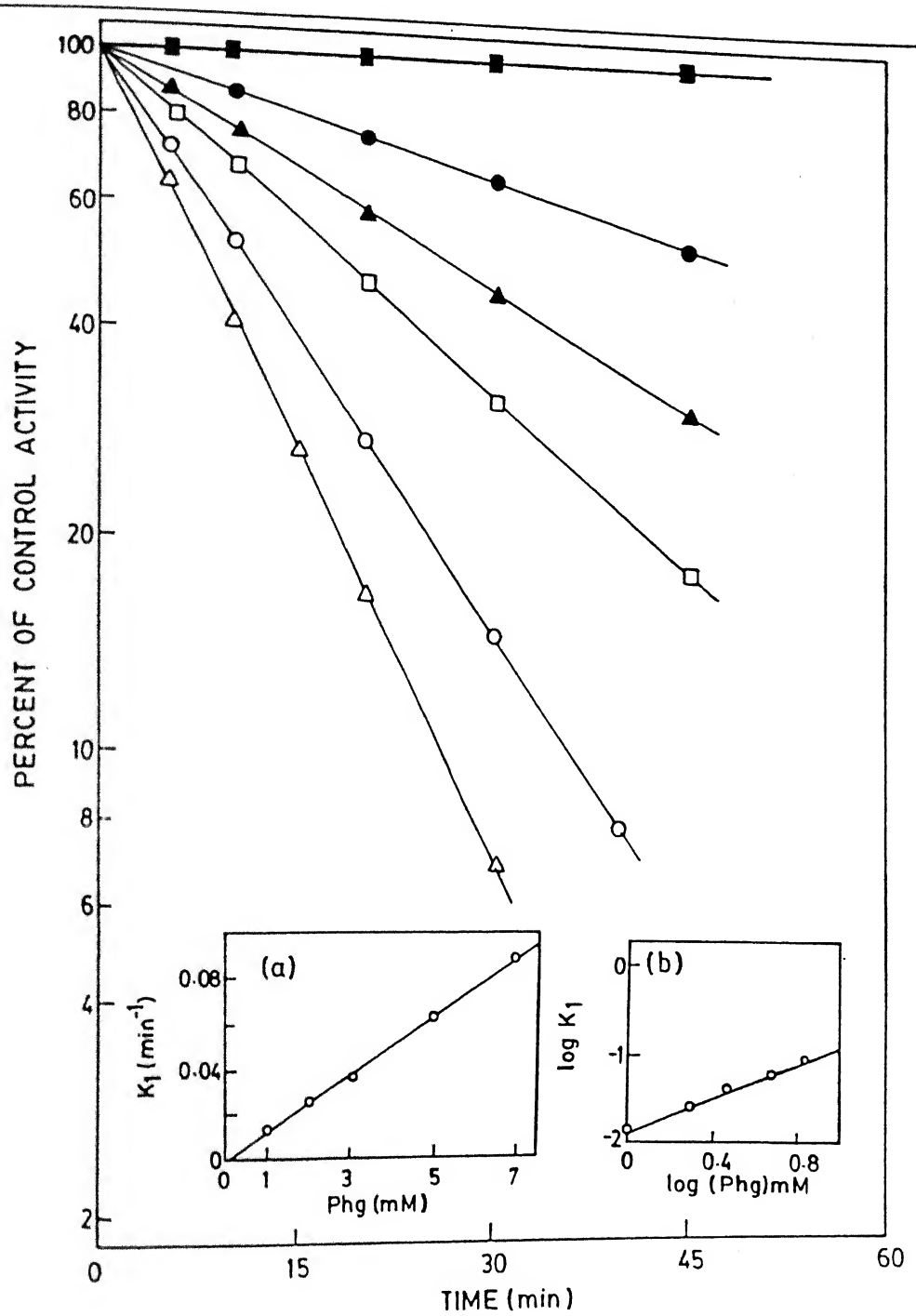

Figure IV.4: *Kinetics of the inactivation of β-ketoacyl reductase activity by phenylglyoxal*. The enzyme (8 μM) in 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT and 80 mM NH_4HCO_3 was incubated with 0(■), 1(●), 2(▲), 3(□), 5(○), and7(Δ)mM phenylglyoxal at 25°C. Aliquots were withdrawn at the indicated time intervals and assayed the residual activity as described in the "Experimental Procedure". Inset **(a)** and **(b)** are linear primary and double log plots of observed pseudo-first order rate constant *vs* phenylglyoxal concentrations.

The reaction of arginine residue with phenylglyoxal as described in Chapter I, section 4.3.2 shows the stoichiometry of 1:2. Double-log plots of k_1 as a function of reagent concentration (Fig IV.2b) and (Fig IV.3 & 4, inset b) were also linear, yielding slopes of 1.10, 1.15 and 1.04, respectively which are close to one indicating, the reaction order of one with respect to the reagent for each of the activities. The second order rate constants for inactivation of β-ketoacyl reductase and enoyl-CoA reductase are nearly identical and the sum of these values equals to the rate constant for the inactivation of overall synthetase activity. This behavior is in accordance with a mechanism whereby phenylglyoxal reacts simultaneously with residue(s) at or near the active-sites of both reductases, and that the modification of either of the reductase domain results in the loss of synthetase activity. This mechanism is also supported by a kinetic order of one with respect to the reagent for loss of synthetase activity. It has been reported in Chapter III, that lysine and histidine residues are present in the enoyl-CoA reductase domain and photomodification studies with PLP indicated that lysine residue is in close proximity of a necleophilic agent. In the present work it was found that arginine is also present in the enoyl-CoA reductase domain. So it is reasonable to consider

that the imidazole group of histidine or guanidinium group of arginine can act as nucleophilic agent in the photoinactivation of FAS by PLP. The proximity of exact amino acid that is present can only be confirmed by determining the amino acid sequence around the NADPH binding site of enoyl-CoA recuctase.

IV.3.2 Effect of substrates and substrate analogs on inactivation of FAS with phenylglyoxal

In order to know the site of inhibition protection studies were performed using substrates and substrate analogs. As shown in (Table IV.1) acetyl-CoA and malonyl-CoA did not show any protection whereas, NADPH or NADP provided significant protection against inactivation of synthetase, β-ketoacyl reductase and enoyl-CoA reductase activities. Similar protection effects were observed with 2'-phosphate derivatives of adenine nucleotides such as 2'-AMP or 2',5'-ADP but not for the 5'-phosphate derivatives viz. 5'-AMP or 5'ADP. NAD^+ had no effect on the loss of synthetase activity. These results suggested that the binding of NADPH to FAS involves specific interaction of the 2'-phosphate group of NADPH with the guanidino group of arginine residue at the active site of the two reductases.

Table.IV.1

The enzyme (12 μM) was incubated with the ligands for 10 min prior to the addition of phenylglyoxal (3 mM). After 30 min the overall FAS and enoyl-CoA and β-ketoacyl reductase activities were determined.

LIGAND	PERCENT OF REMAINING ACTIVITY		
	FAS	β-KETOACYL REDUCTASE	ENOYL-CoA REDUCTASE
None	14	32	37
Acetyl-CoA (1 mM)	14	–	–
Malonyl-CoA (1 mM)	15	–	–
NAD^+ (5 mM)	12	32	35
2'-AMP (5 mM)	47	70	67
2',5'-ADP (5 mM)	51	78	75
NADPH (5 mM)	54	79	75

IV.3.3 Modification of FAS by 2,3-butanedione

2,3-butanedione, another widely used reagent, which in borate buffer modifies arginine residues in proteins [5,18], was also used for modification of FAS. The inactivation was dependent on time and concentration of the reagent (Fig. IV.5). The inactivation followed pseudo-first order kinetics, as

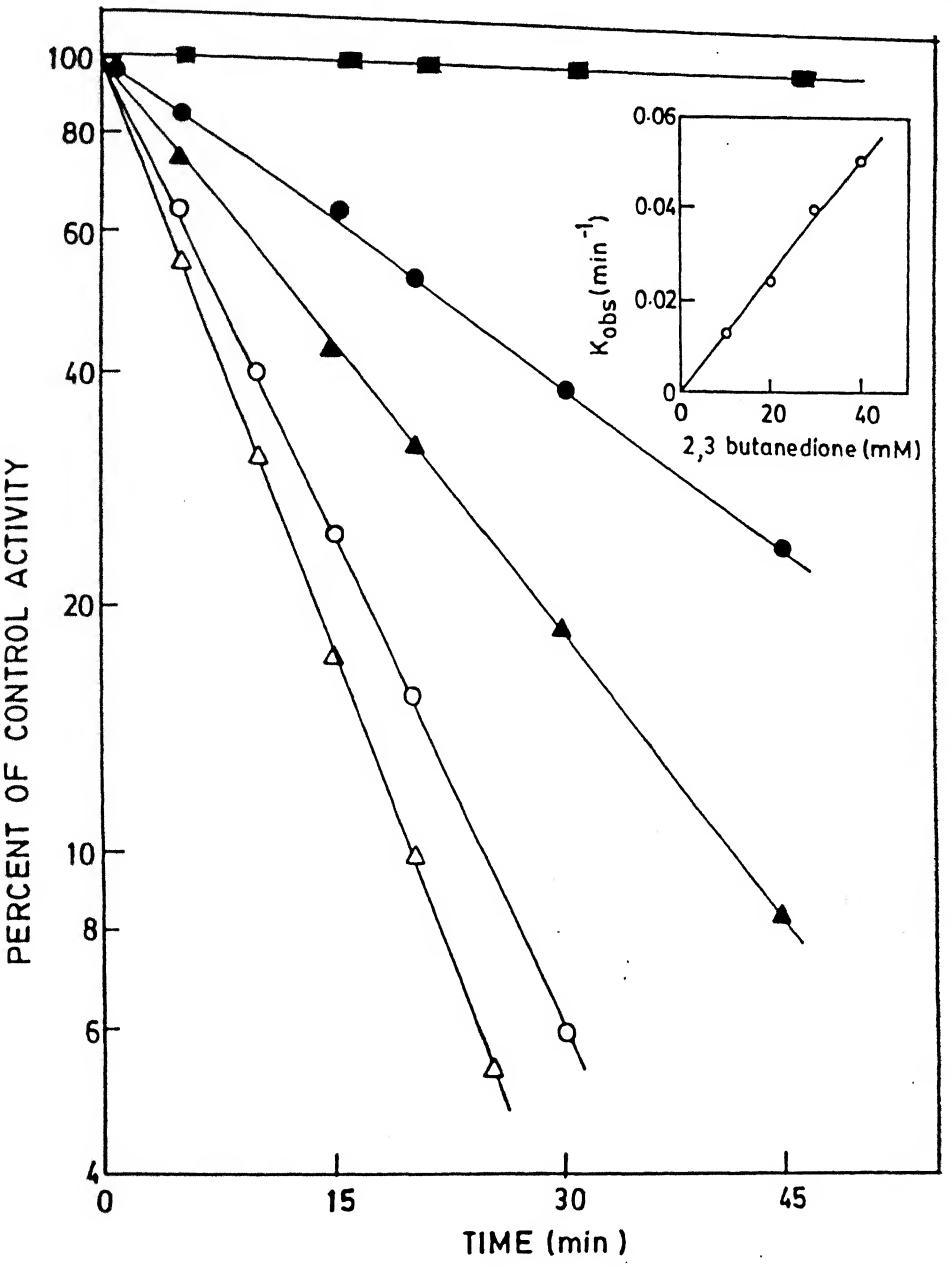

Figure IV.5: *Kinetics of the inactivation of the of overall FAS by 2,3-butanedione* The enzyme (3 μM) in 20mM borate buffer (pH 7.5) containing 200 mM KCl, 1 mM EDTA and 1 mM DTT was incubated with 0(■), 10(●), 20(▲), 30(o), and 40(Δ) mM 2,3-butanedione at 25°C. Aliquots were withdrawn at the indicated time intervals and assayed the residual activity as described in the "Experimental Procedure". The inset shows a plot of observed pseudo-first order rate constant *vs* 2,3-butanedione concentrations.

indicated by semi-log plots of activity versus time which was linear. The second order rate constant (k_2) was obtained from slopes of linear plots of pseudo-first order rate constants (k_1) *vs* reagent concentration (Fig. IV.5,inset) and was $1.2x10^{-3}mM^{-1}min^{-1}$. 2,3-butanedione also inactivated β-ketoacyl and enoyl-CoA reductase activities of FAS but the overall FAS activity was affected more than the two reductase activities (Fig.IV.6).

IV.3.4 Effect of substrates and substrate analogs on FAS inactivation by 2,3-butanedione

The effect of substrates and substrate analogs were studied to determine the site of inactivation by 2,3-butanedione. Saturating concentration of substrate-ligands were used to achieve full-site occupancy. As shown in (Table IV.2), the cofactor NADPH afforded maximum protection against the inactivation of synthetase, β-ketoacyl reductase as well as enoyl-CoA reductase activities. Similar protection effects were observed with 2'-phosphate derivatives of adenine nucleotides such as 2'-AMP or 2'5'-ADP, and not with the 5'-phosphate derivatives viz. 5'-AMP or 5'ADP. NAD^+ had no effect on the loss of synthetase activity. Acetyl-CoA and malonyl-CoA were found to be ineffective in protecting the

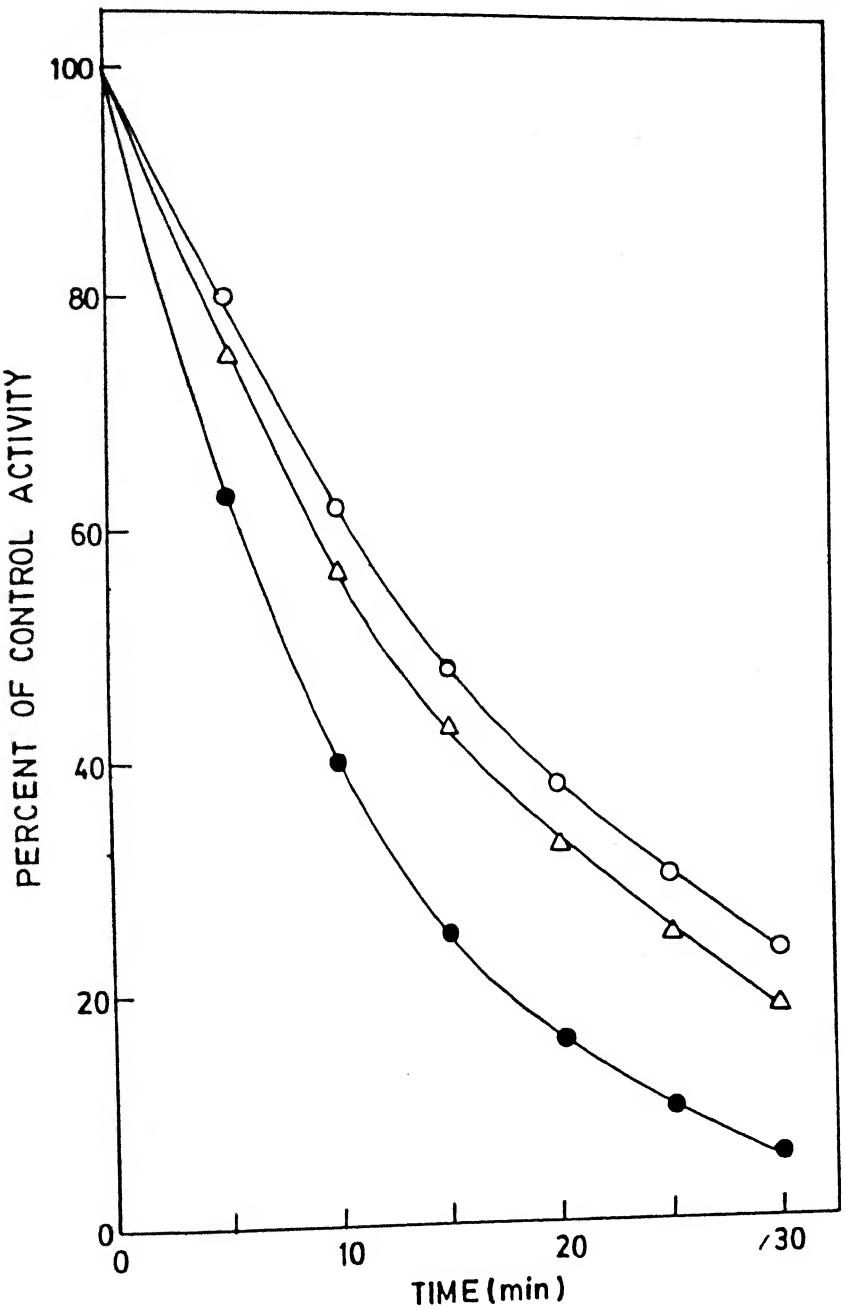

Figure IV.6: *Time course of inactivation of overall, enoyl-CoA reductase and β-ketoacyl reductase of FAS by* 2,3-*butanedione.* Incubation with 2,3-butanedione was carried out at a protein concentration of 3, 12 and 8 μM for overall (●), enoyl-CoA reductase (Δ) and β-ketoacyl reductase (o) activity respectively with 30 mM 2,3-butanedione in the reaction medium. Aliquots were taken at time intervals and assayed the residual activity as described in the "Experimental Procedure".

enzyme against inactivation. These results suggested that the binding of NADPH to FAS involves specific interaction of the 2'-phosphate with the guanidino group of arginine residue at the active site of the two reductases.

Table IV.2

The enzyme (3 mg/ml) was incubated with the ligands for 10 min prior to the addition of (30 mM) 2,3-butanedione. After 30 min the overall FAS and enoyl-CoA and β-ketoacyl reductase activity was determined.

LIGAND	PERCENT OF REMAINING ACTIVITY		
	FAS	β-KETOACYL REDUCTASE	ENOYL-CoA REDUCTASE
None	6	20	17
Acetyl-CoA (1 mM)	7	–	–
Malonyl-CoA (1 mM)	6	–	–
NAD^+ (5 mM)	5	19	15
5'-AMP (5 mM)	6	21	14
2'-AMP (5 mM)	48	62	58
2',5'-ADP (5 mM)	54	70	64
NADPH (5 mM)	52	71	65

IV.3.5 Effect of pH on FAS inactivation by phenylglyoxal

The determination of the rate of modification of an enzyme by an inhibitor as a function of pH allows one to calculate the pk_a of the reactive group in an enzyme. The inactivation pattern of FAS by phenylglyoxal in the pH range of 6.0-8.0 is shown in (Figure IV.7). A plot of log of percent control activity *vs* time gave a straight line. The apparent second order rate constants obtained as described in section 2.2.7 were plotted as a function of pH (Fig. IV.7, inset a). The data from the linear plot was used for the determination of pk_a of the reacting residue in phenylglyoxal reaction that is shown in (Fig. IV.7, inset b) and the pk_a value of 7.5 was obtained. This pk_a value is comparatively lower than that of 9.5 for arginine. This might be due to specific hydrophobic environment at the active site. Phenylglyoxal can react with the guanidino group of arginine residue in the pH range, 7.0 to 8.0 [19]. The derivative, which contains two phenylglyoxal moieties per guanidino group, is sufficiently stable, particularly under mildly acidic conditions (around pH 4.0). At neutral or alkaline pH the derivative gets decomposed slowly [19].

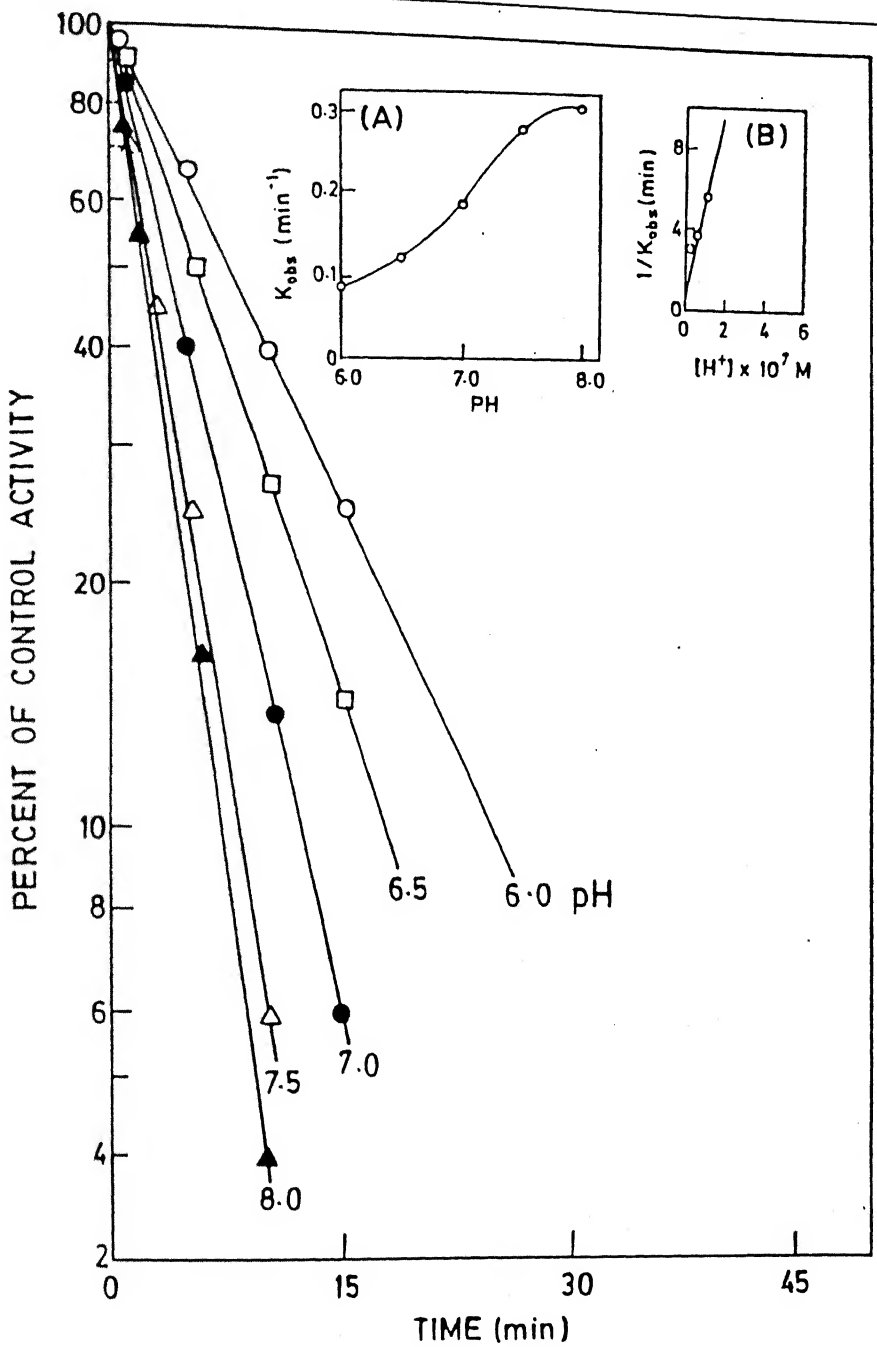

Figure IV.7: *Fatty acid synthetase inactivation by phenylglyoxal as a function of pH.* The enzyme (3 μM) was inactivated with 7mM PLP in 0.2 M potassium phosphate buffer over a pH range of 6.0-8.0. At indicated time the aliquots were removed and enzyme activity was determined. Inset (A): Plot of dependence of pseudo-first order rate constant of inactivation on pH. Inset (B): $1/k_{obs}$ min *vs* $[H^+]$ [reploting the data of (A)].

IV.3.6 Number of essential arginine residue(s) modified by phenylglyoxal

Although phenylglyoxal is highly specific for arginine residue [20], other amino acids such as cysteine and to a lesser extent histidine and lysine residues are also known to undergo reaction with phenylglyoxal under certain conditions [13]. In a separate experiment, the essential SH groups of FAS were reversibly blocked by incubating 3 μM enzyme with 30 μM 5,5'-dithiobis(2-nitrobenzoic acid) for 35 s at room temperature. Following removal of the excess DTNB by gel filtration, the enzyme derivative was reacted with 2 mM phenylglyoxal and the aliquots from that mixture were assayed at different time intervals for synthetase activity 30 min after the addition of 50 mM DTT. The pseudo-first order rate constant of inactivation of 0.02 min^{-1} was comparable to that obtained for the unblocked enzyme (k_1=0.0198 min^{-1}), indicating that phenylglyoxal inactivation did not involve reaction of essential SH groups. In order to determine the number of reactive residues involved in inactivation the absorbance of the diphenylglyoxal adduct with guanidino group of arginine of enzyme molecule was measured at 250 nm at different stages of modification. As shown in (Fig. IV.8) a plot of residual synthetase activity *vs* arginine residues modified was linear

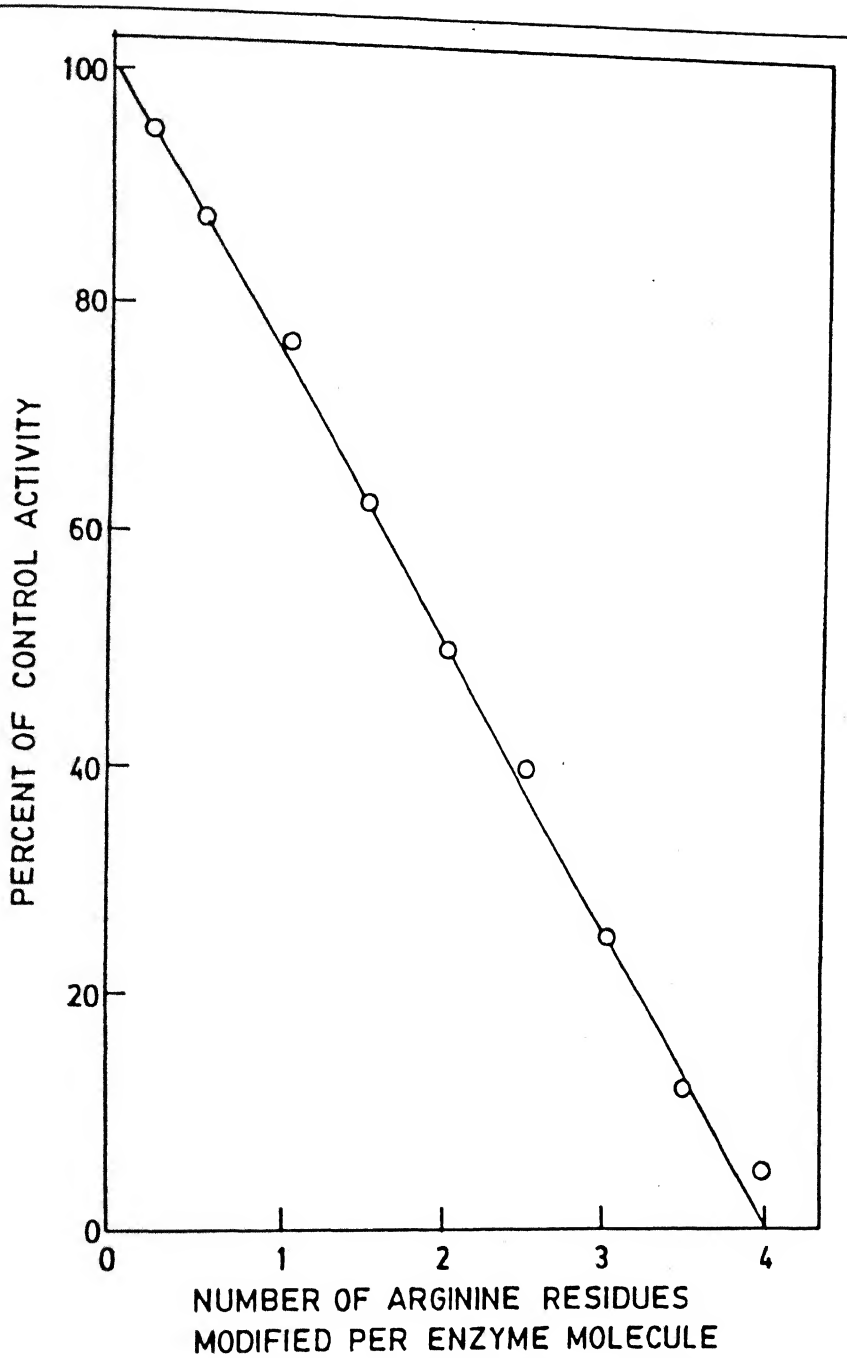

Figure IV.8: *Stoichiometry of inactivation of FAS by phenylglyoxal.* The enzyme (3 μM) was incubated with 2.5 mM of phenylglyoxal under the reaction conditions as described in the "Experimental Procedure". The modified enzyme was passed through a column according to Penefsky to remove the excess reagent. The concentration of diphenylglyoxal adduct was determined by absorbance at 250 nm using an extinction coefficient of 11,000 $cm^{-1}M^{-1}$. In a parallel experiment the aliquots were withdrawn from the incubation mixture at different time intervals and added to 10 mM of arginine to terminate the reaction and assayed for FAS activity.

and an extrapolation gave a value of 4.0, indicating that four arginine residues per enzyme molecule reacted for the complete loss of activity. Therefore it appearsthat the four arginine residues modified are at the two reductase sites. It is reasonable to conclude that there is one essential arginine at each of the β-ketoacyl reductase and enoyl-CoA reductase sites per subunit. The presence of fast-reacting, non-essential residues as in the synthetase from goose uropygial gland [7] was not observed, indicating greater selectivity of reagent. This stoichiometric results were similar to that reported for chicken liver FAS [8].

IV.4 Summary

Two arginine modifying reagents, phenylglyoxal and 2,3-butanedione inactivated overall FAS, β-ketoacyl and enoyl-CoA reductase activity. The inactivation was irreversible and dependent on the concentration of inhibitor and the time. Modification of FAS with these reagents followed a pseudo-first order kinetics. The second-order rate constants for inactivation of β-ketoacyl reductase and enoyl reductase were nearly identical and the sum of these values was equal to the rate constant for the inactivation of overall synthetase activity. These inactivations were partially prevented by NADPH and 2′-phosphate derivatives of adenine nucleotides such as

2′-AMP or 2′,5′-ADP, but not by the 5′-phosphate derivatives 5′-AMP or 5′-ADP and CoA thioesters like acetyl-CoA or malonyl-CoA. These results suggested that binding of NADPH to FAS involves specific interaction of the 2′-phosphate with the guanidino group of arginine residue at the active site of the two reductases. Stoichiometric results showed that four moles of arginine residues were modified per mole of enzyme by phenylglyoxal upon complete inactivation. The results of the present chemical modification studies are consistent with the previous findings on animal FAS that each subunit of FAS contains one β-ketoacyl reductase and one enoyl-CoA reductase and further suggest that one essential arginine residue is present at each of these active sites.

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CHAPTER V

STUDIES ON THE REACTIVITY OF THE ESSENTIAL SULFHYDRYL GROUPS OF PIGEON LIVER FATTY ACID SYNTHETASE USING THIOL SPECIFIC REAGENTS AND O-PHTHALALDEHYDE, A BIFUNCTIONAL REAGENT

V.I. INTRODUCTION

The pigeon liver fatty acid synthetase, a multifunctional protein having two polypeptides catalyzes the formation of palmitic acid through successive elongation of acetyl-CoA as primer by seven two-carbon acyl groups from malonyl-CoA in the presence of NADPH [1]. Earlier work by Jacob Butterworth, & porter [2] , Chesterton et.al [3,4] and Philips et.al [5] has shown, through the peptide mapping, the presence of three chemically distinct covalent binding sites for acetyl and two for malonyl moieties in the complex. Site (A_2) 4'-phosphopantetheine and a nonthiol site (B_1) bind both acetyl and malonyl groups, while another sulfhydryl site, B_2

(cysteine) binds only an acetyl group [5]. Fatty acid synthesis is initiated by the transfer of an acetyl moiety of acetyl-CoA to the B_1 site followed by successive transfers to A_2 and then to B_2 site [6,7]. The malonyl group is similarly transferred from B_1 to the A_2 site. Condensation between the acetyl moiety on the B_2 site of the enzyme and the malonyl group bound to the A_2 site results in the formation of acetoacetyl- 4'-phoshopantetheine enzyme, with simultaneous release of CO_2. Previously, it was found that some sulfhydryl reagents like N-ethylmaleimide, p-chloromercuribenzoate, Iodoacetamide, sodium arsenite have inhibitory effects on fatty acid synthesis by blocking acetyl-CoA and/or malonyl-CoA binding sites and iodoacetamide blocks specifically β-ketoacyl synthase sulfhydryl group (B_2 site) [8].

In the present study, a detailed investigation on the effect of 5,5'-dithiobis-(2-nitro benzoic acid) [DTNB] on FAS, which leads to inactivation and subsequent cross linking of subunits, has been carried out under different experimental conditions. o-Phthalaldehyde, a fluorogenic bifunctional compound has been found to be an important reagent in a number of quantitative and qualitative studies [9-14]. The use of o-phthalaldehyde as an active site reagent was characterized by Palaczewski et al [15]. It forms an isoindole derivative by

cross linking ε-amino group of lysine and sulfhydryl group of cysteine residue located not more than 3Å apart from each other [16]. Lysine and cysteine residues have been identified as active site residues in various enzymes by the use of o-phthalaldehyde [17-22]. In chapter IV it has been reported that lysine residue is essential for synthetase activity. In the present study it has been shown that phosphopantetheine-SH group is involved in the reaction of o-phthalaldehyde and DTNB but the lysine residue involved in o-phthalaldehyde reaction is non-essential.

V.2 EXPERIMENTAL PROCEDURE

V.2.1 Materials

5,5'-dithiobis-(-2-nitro benzoic acid) [DTNB], o-phthalaldehyde (OPA), Acetyl-CoA, Malonyl-CoA, NADPH, sephadex G-50, Buffer components were perchased from Sigma Chemical Company, (USA). Dithiothreitol [DTT], was obtained from Cal Biochem. All other chemicals were of highest purity grade commercially available.

V.2.2 Methods

V.2.2.1 Enzyme preparation and assay

Pigeon liver fatty acid synthetase was prepared and assayed as described in chapter II section 2.3 and 2.5. The

enzyme was thawed, incubated at 25°C for 2h before use, in order to permit full activation and equilibrated with the appropriate thiol free medium by the column centrifugation method using sephadex G-50 [23]. The enzyme preparation was freed from detectable DTT contamination since it causes rapid reactivation of the inactivated enzyme during the assay in presence of thiol reactive reagent. In control experiment, it was shown that removal of thiol compound has no effect on the enzyme activity.

V.2.2.2 Inactivation of FAS by thiol specific reagent DTNB

The inactivation of FAS was carried out under low salt and high salt conditions. The pigeon liver FAS (2.6 μM) was treated with the varying concentrations of DTNB (4-12 μM) under low salt condition in 6 mM EDTA, 1% (v/v) glycerol at pH 7.0 and 25°C. The inactivation under high salt condition was carried out in 200 mM of potassium phosphate buffer in above mentioned reaction mixture. At different time intervals aliquots were withdrawn and assayed for the residual FAS activity. A control without DTNB was run concurrently.

V.2.2.3 Effect of salts on inactivation of FAS by DTNB

FAS enzyme (2.6 μM) was incubated with different salts,

potassium phosphate, KCl and Tris-Cl in the concentration range of (25-200 mM) with DTNB (4 μM) containing 6 mM EDTA, 1% (v/v) glycerol at pH 7.0 and 25°C. In other experiment FAS enzyme (2.6 μM) was treated with 6 μM of DTNB at the lowest salt concentration i.e. 3 mM EDTA, 1% (v/v) glycerol). At different time intervals aliquots were taken for the determination of enzyme activity. The enzyme under same reaction conditions without DTNB was used as control.

V.2.2.4 Effect of substrates on inactivation of FAS by DTNB

Protection experiments were carried out under low and high salt conditions. In low salt, 2.6 μM of FAS was incubated with 8 μM of DTNB in 6 mM EDTA, 1% (v/v) glycerol at pH 7.0 and 25°C. Experiment with high salt was carried out under the same conditions except that 200 mM of potassium phosphate was added to the reaction mixture. Substrates like acetyl-CoA, malonyl-CoA and NADPH were added to the incubation mixture 15 min prior to the addition of DTNB. The reaction was allowed to proceed for 5 min and (30 s) under high salt and low salt conditions respectively. The enzyme preincubated with the substrates without DTNB was taken as control.

V.2.2.5 Reactivation of DTNB-inactivated FAS by DTT

The pigeon liver FAS (2.6 μM) was incubated with 8 μM of DTNB in 6 mM EDTA, 1% (v/v) glycerol at pH 7.0 and 25°C for different time intervals (45, 60, 120 and 180 s) before adding 40 mM of DTT. The time course of reactivation of FAS activity was determined.

V.2.2.6 Cross linking studies

The pigeon liver FAS (10 μM) was incubated with DTNB in 6 mM EDTA, 1% (v/v) glycerol, pH 7.0 ,at 25°C, in the DTNB :enzyme ratio of 2:1 for the incubation periods of 60 s, 120 s and 180 s and in the ratio of 9:1 for the periods of 180 s and 600 s. The reaction was stopped by the addition of sodium dodecyl sulfate buffer and quickly heated. The samples containing 50 μg of enzyme were electrophoresed.

V.2.2.7 SDS polyacrylamide gel electrophoresis of DTNB-modified enzyme

Vertical slab gel electrophoresis was carried at ambient temperature according to the method of Laemmli [24], as described in the Chapter II, section II.2.4 at a sodium dodecyl sulfate concentration of 0.1%. The acrylamide concentrations were 5% for the separating gel and 3.9% for the stacking gel.

To each enzyme sample, 4 volumes of sodium dodecyl sulfate buffer containing sodium dodecyl sulfate, 2%; Tris-Cl, 62.5 mM, glycerol, 1%(v/v) and 0.05% (w/v) bromophenol blue, pH 6.8, was added and immediately heated in a boiling water bath for 2 min. The electrophoresis was carried out at 2.5 mA current per lane for 6-8 h.

V.2.2.8 Stoichiometry of DTNB-modified FAS

The FAS (2.6 μM) was incubated with varying concentrations of DTNB (0-4 μM) in 0.2 M potassium phosphate buffer (pH 7.0) containing 6 mM EDTA and 1% glycerol (v/v) at 25°C. The residual activity was estimated after completion of the reaction (10 min). The number of essential SH groups modified was determined by a plot of residual activity versus concentration of DTNB per enzyme mlecule

V.2.2.9 Inactivation of FAS by Iodoacetamide

The time course for the inhibition of the pigeon liver FAS by iodoacetamide was determined by reacting the enzyme (1 mg/ml) at 25°C with 0.5 mM iodoacetamide in 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. At various time intervals, samples were withdrawn and assayed for FAS activity. Inhibition of the synthetase by iodoacetamide in the presence

of acetyl-CoA (0.5 mM) or malonyl-CoA (0.5 mM) was also carried out.

V.2.2.10 Inactivation of FAS by o-phthalaldehyde

The solution of o-phthalaldehyde (OPA) was prepared in 0.2 M phosphate buffer (pH 7.0) containing 1% methanol which did not show any effect on the enzyme activity. The enzyme (8 μM) in 0.2 M potasium phosphate buffer (pH 7.0) was incubated with varying concentrations (8-24 μM) of o-phthalaldehyde at 25°C. At different time intervals aliquots were withdrawn and assayed for overall and enoyl-CoA reductase activities.

V.2.2.11 Effect of substrates on inactivation of FAS by o-phthalaldehyde

For the protection experiments, the enzyme (8 μM) was incubated with the substrates for 15 min before the addition of o-phthalaldehyde (16 μM) in 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA at 25°C. The residual activity was measured as described earlier in Chapter II section 2.5. The controls without o-phthalaldehyde were run concurrently.

V.2.2.12 Spectral studies on o-phthalaldehyde reaction with FAS enzyme

Fatty acid synthetase (4 mg/ml) was incubated with 16 μM of o-phthalaldehyde at 25°C for 30 min under the low salt and high salt conditions. Fluorescence emission spectra of isoindole derivative resulting from synthetase-o-phthalaldehyde reaction were recorded by excitation at wave length 337 nm on luminescence spectrometer, Perkin Elmer (Model LS 50B). The fluorescence was recorded in cuvettes of 1 cm light path at 25°C. Similarly fluorescence spectra of o-phthalaldehyde reaction with the FAS in presence of substrates were recorded.

V.2.2.13 Stoichoimetry of the reaction of FAS with o-phthalaldehyde

The stoichiometry of reaction was determined by incubating the enzyme (4 mg/ml) in 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA at 25°C with 20 μM o-phthalaldehyde for 30 min. The number of mol of o-phthalaldehyde incorporated per mol of enzyme was determined by taking the ratio of concentrations of isoindole derivative and enzyme. The concentration of isoindole derivative was determined using absorbance coefficient of 7660 $M^{-1}cm^{-1}$ at 337 nm [10] and the concentration of enzyme was determined by the method of Lowry

et al. method [25]. The molecular weight of FAS was taken as 450,000.

V.2.2.14 Effect of DTNB, iodoacetamide, and TNBS preincubation of FAS on o-phthalaldehyde binding

The enzyme (2.6 μM) was incubated with 4 μM of DTNB for 30 s and 5 min prior to incubation with 24 μM o-phthalaldehyde for 30 min in 6 mM EDTA, 1% (v/v) glycerol (pH 7.0) at 25°C. In another set the enzyme was preincubated with 0.5 mM iodoacetamide for 30 min before addition of 24 μM o-phthalaldehyde. Similarly the enzyme was first incubated with 1 mM 2,4,6-trinitrobenzenesulphonic (TNBS) in 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA at 25°C for 30 min followed by incubation with 24 μM o-phthalaldehyde for 30 min. The effect of preincubation of these inhibitor on the reaction of enzyme with o-phthalaldehyde was characterized by monitoring the fluorescence emission spectra.

V.2.2.15 Double inhibition studies on FAS using DTNB and o-phthalaldehyde

The enzyme (2·6μM) was incubated with DTNB (4 μM) in 6 mM EDTA, 1% (v/v) glycerol pH 7.0 at 25°C for 30 s, followed by 24 μM o-phthalaldehyde for 1 min or 24 μM o-phthalaldehyde for 1

min followed by 4 μM DTNB for 30 S. At the end of indicated treatments the residual activity was measured. To study the reactivation of FAS activity with DTT the remaining incubation mixtures were equilibrated with the above buffer containing 0.2 M potassium phohphate by column-centrifugation to remove excess DTNB and o-phthalaldehyde according to the method of Penefsky [23]. Eluate was allowed to react with DTT (40 mM) for 5 min. The recovery of activity was then determined. Control sets in the absence of inhibitor or in presence of only one inhibitor treated with same concentrations of DTT were run concurrently.

V.3 Results and discussion

V.3.1 Kinetics of inactivation of FAS by DTNB

Pigeon liver FAS was rapidly and reversibly inactivated by DTNB. The rate of inactivation was affected by salt concentration. The inactivation followed bimolecular process according to the equation 1, as the reagent concentration is not in large excess over the protein concentration the assumption of pseudo-first order kinetics is not valid,

$$k_2(A-B)xt = \ln\{[B(A-P)]/[A(B-P)]\} \text{ ------eq(1).}$$

Where, k_2 is the second order rate constant. A and B are the initial concentrations of DTNB and fatty acid synthetase,

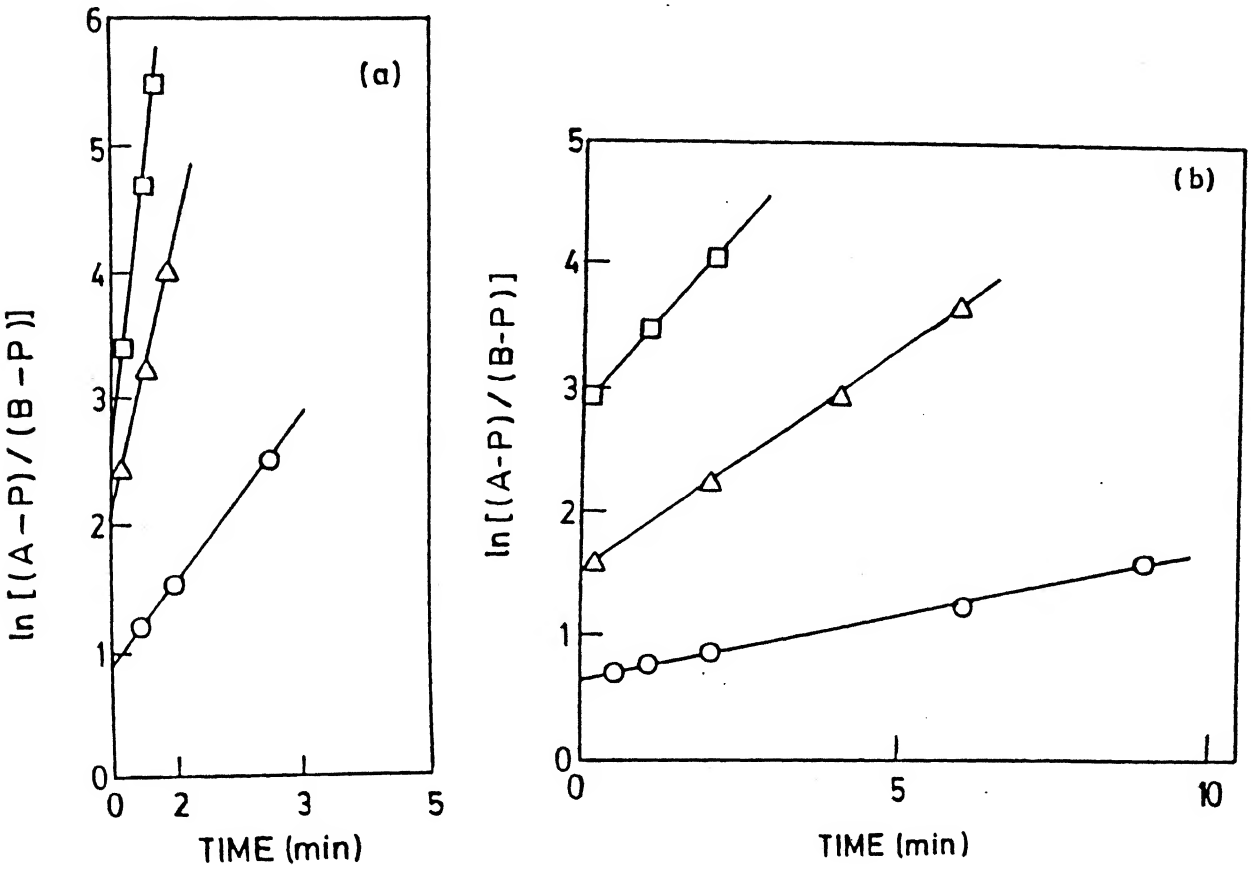


Figure V.1: *Second order plot on the inactivation of FAS by DTNB under the high salt and low salt condition.* The enzyme (2.6 μM) was incubated with 4(○), 8(Δ) and 12(□) μM of DTNB under **(a)**low salt condition containing 6 mM EDTA and 1% (v/v) glycerol at pH 7.0 and 25°C and **(b)** high salt inactivation was carried out in 0.2 M of potassium phosphate buffer containing the same reaction mixture. Aliquots were taken at indicated time intervals and assayed the residual activity as described in the "Experimental Procedures".

respectively. P is the concentration of inactivated enzyme and equals B xfractional activity loss at time t. A plot of ln(A-P)/(B-P) versus time yieldeda straight line with a slope of k_2(A-B) from which k_2 was calculated as ($7.0^{\pm}0.26$ $mM^{-1}s^{-1}$) at 6 mM EDTA, 1% glycerol (v/v), pH 7.0 and at 25^{o}C. The rate was decreased in the presence of 200 mM potassium phosphate to ($1.1^{\pm}0.06$ $mM^{-1}s^{-1}$) as shown in (Fig. V.1.a & b) respectively.

V.3.2 Effect of salts on the rate of inactivation of FAS by DTNB

The rate of inactivation of FAS by DTNB was highly dependent on salt concentration. Inactivation of the synthetase by DTNB was inhibited by increasing the salt concentration. The rate constants of inactivation were determined at different concentrations of potassium phosphate, KCl and Tris-Cl. As shown by a plot of k_2 versus salt concentration in (Fig. V.2), the inactivation was maximum (k_2= 9.0 $mM^{-1}s^{-1}$) at the lowest salt concentration of 3 mM EDTA and 1% (v/v) glycerol. Increasing the molarity of potassium phosphate in reaction medium containing 6 mM EDTA and 1% (v/v) glycerol resulted in an asymptotic decrease of the rate constant from 7.0 to a value of 1.1 $mM^{-1}s^{-1}$. Furthermore, this salt effect was nonspecific as the rate constant obtained with other salts were almost

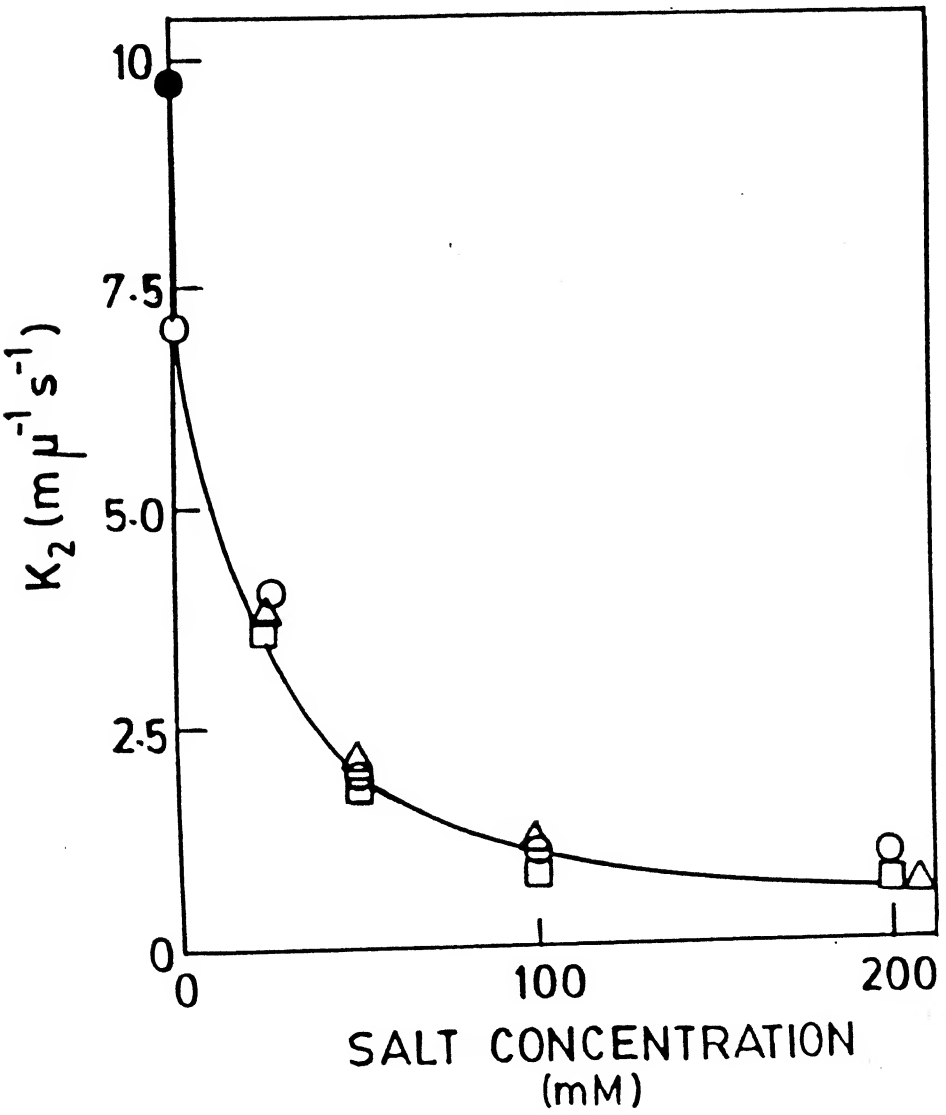


Figure V.2: *The dependence of the second order rate constant of inactivation on salt concentration.* The enzyme (2.6 μM) was incubated with 4 μM of DTNB in 6 mM EDTA, 1% (v/v) at pH 7.0 and 25°C and salt as indicated. The salts are (o), potassium phosphate; (Δ) KCl; and (□), Tris-cl. The filled circle represents the rate constant obtained at 3 mM EDTA, 1% (v/v) glycerol without added salt.

identical, suggesting that it is the change in the ionic strength of the incubation medium which is responsible for the decrease of rate constant of inactivation. The effect of salts on reactivity was unique for the essential SH groups of fatty acid synthetase as in control experiments, the second order rate constant of cysteine with DTNB as determined by following trinitrobenzene (TNB) derivative formation at 412 nm was identical in low salt and high salt conditions. The strong dependence of reactivity of -SH group on salt concentration could be attributed to salt induced conformational changes affecting the local environment of this group. A plausible model involving two conformational states of the enzyme is shown in (Fig. V.3). For the purpose of clarity, only a single site comprised of a cysteine -SH group from subunit A and a phosphopantetheine -SH group from subunit B is shown for each conformer. C and R are the condensation and reductase domains, respectively, and ACP is the acyl carrier protein component. In conformer I, the DTNB (D) bound noncovalently to subunit A is poised for reaction with the adjacent phosphopantetheine -SH group on subunit B, which in turn cross-links with the cysteine -SH group on A. Such a structural arrangement, however, is absent in conformer II. At very low salt concentration the enzyme exists solely as conformer I, in which the

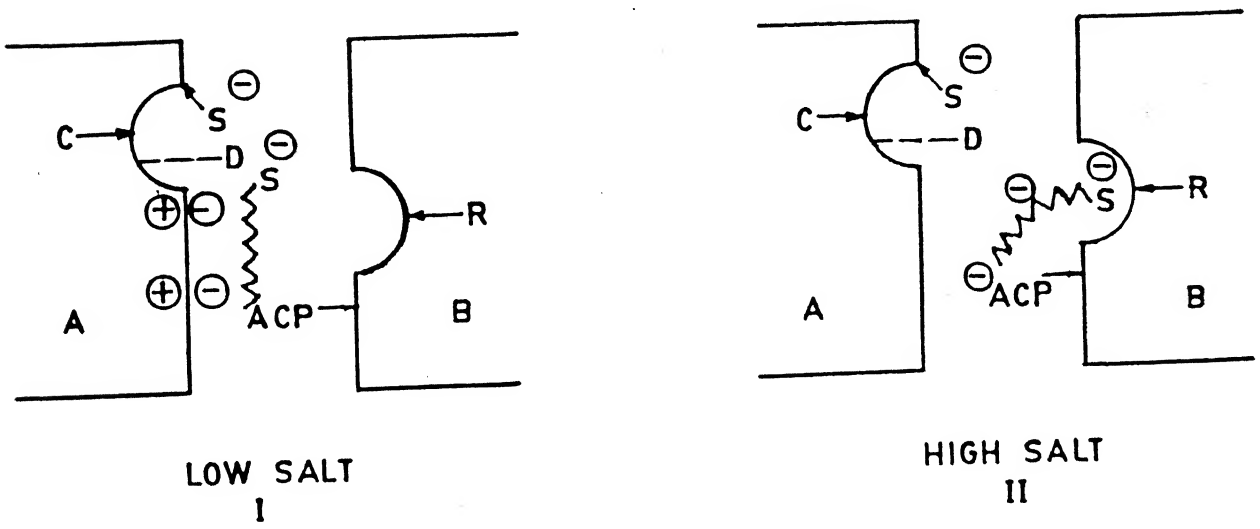


Figure V.3: Possible arrangements of phosphopantetheine and cysteine -SH groups in fatty acid synthetase conformers

phosphopantetheine SH group is highly reactive. The addition of salt induces a transition to conformer II, and the reactivity of this group is markedly reduced.

V.3.3 Effect of substrates on the inhibition of FAS activity by DTNB

In order to determine the binding site of DTNB in pigeon liver FAS, protection experiments were carried out. The effect of different substrates on the inactivation of FAS under the low salt and high salt conditions are shown in Table V.1. Under both these conditions, the inactivation against DTNB was protected by acetyl-CoA or malonyl-CoA. Since both the acyl-CoA substrates bind to the phosphopantetheine -SH group [8,26], the protective effect by these substrates suggested that DTNB reacts with the SH group of phosphopantetheine. Significant protection was also observed with NADPH. However, the protection effect by nucleotide was less pronounced at high salt concentration. At either salt concentrations, combinations of acetyl- (or malonyl-) CoA and NADPH provided nearly complete protection against inactivation by DTNB. In fatty acid synthetase reaction, the pyridine nucleotides bind to reductase sites on each subunit. The protection afforded by NADPH might be due to the ligand-induced conformational change of the

enzyme molecule affecting the reactivity of the susceptible SH group.

Table V.1

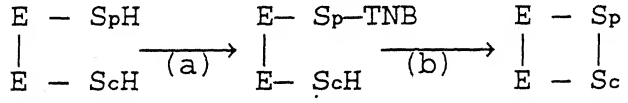
Effect of substrates on DTNB inactivation of FAS

The enzyme (2.6 μM) was preincubated with indicated concentrations of different substrates under high salt and low salt conditions (as described im the experimental procedure) for 15 min. The mixtures were treated with 8 μM of DTNB for 5 min and 30 s for high salt and low salt samples, respectivly. The remaining activity was measured as described in section V.2.

LIGANDS	PERCENT OF CONTROL ACTIVITY	
	LOW SALT	HIGH SALT
None	6	10
Acetyl-CoA (200 μM)	75	72
Malonyl-CoA (200 μM)	73	70
NADPH (1 mM)	40	29
Acetyl-CoA (200 μM) +NADPH (1 mM)	97	95
Malonyl-CoA (200 μM) +NADPH (1 mM)	95	94

V.3.4 Cross linking studies

Pigeon liver FAS was inactivated by DTNB and incubated for additional periods to permit cross linking between subunits. Intersubunit cross linking can be shown as a two step reaction in a scheme as described for chicken liver FAS inactivation by DTNB [27].



I II III

Scheme-I

E denotes the enzyme subunit, and SpH and ScH are phosphopantetheine and cysteine SH groups. In this scheme, steps (a) and (b) represent the inactivation of enzyme by reaction of the phosphopantetheine SH group with DTNB and subsequent dimerization, respectively. The results of SDS-polyacrylamide gel electrophoresis of DTNB modified FAS are shown in (Fig.V.4). In the control, enzyme sample without DTNB (lane B in Fig.V.4 and in experiment I Table V.2), only the monomer band was observed. The time course experiments (lanes C-G in experiments I and II) showed that while inactivation occured in 15-30 s, cross linking required 2-3 min, due to the slower

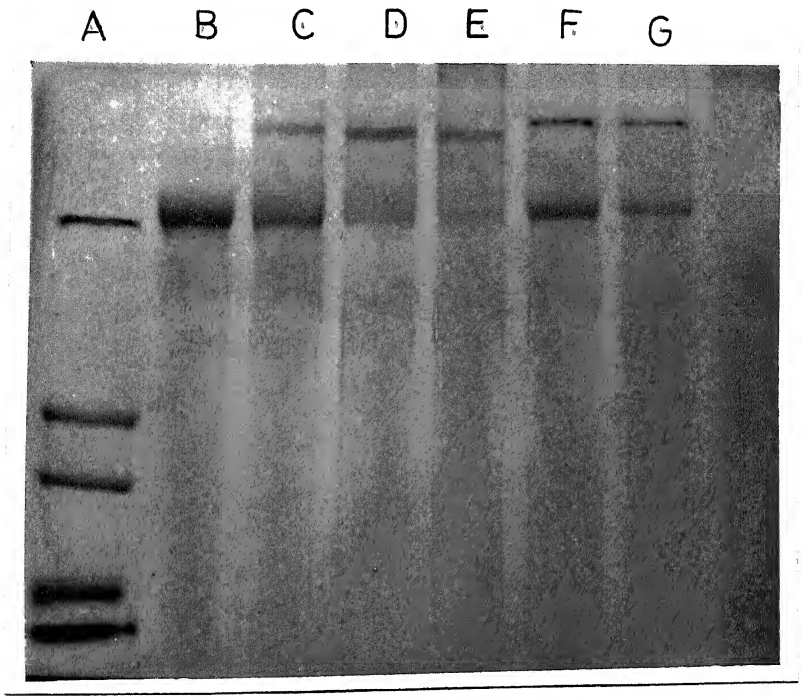


Figure V.4: *Sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of DTNB-modified enzyme.* The enzyme (10 μM) was incubated with DTNB in 6 mM EDTA, 1% (v/v) glycerol, pH 7.0 ,at 25°C, with different DTNB :enzyme ratio. For details see "Experimental Procedure". Lane A is representing the Bio-Gel protein standards and molecular weights (in descending order), myosin,(200 kDa)., β-galactosidase,(116 kDa)., phosphorylase a, (97 kDa)., bovine serum albumin,(66 kDa)., ovalbumin,(45 kDa). Lane B is the control enzyme sample without DTNB. Lane (C-G) is showing the time course of cross linking at different DTNB:enzyme ratio.

dimerization step. The cross linking in the lane C was lower than the maximum cross linking obtained in the lane D and E.

Table.V.2

The effects of incubation time and additions on cross linking

The incubations contained 10 μM of FAS and DTNB and other additions in amounts indicated in 6 mm EDTA, 1% glycerol (v/v), pH 7.0, T= 25°C. The reaction was stopped by the addition of SDS buffer and quickly heated, and samples containing 50 μg of enzyme were electrophoresed as described in the experimental procedure.

EXPERIMENT	DTNB: ENZYME RATIO	PRIMARY INCUBATION WITH DTNB		SECONDARY INCUBATION WITH DTNB		CROSS LINKING
		*EFECTOR	TIME (s)	EFECTOR	TIME (s)	
I	2:1	None	60	None	0	Cross linking was increased with time
		None	120	None	0	
		None	180	None	0	
II	9:1	None	180	None	0	Cross linking was reduced with time
		None	600	None	0	

*EFFECTOR may be salt and substrates (Acetyl-CoA, Malonyl-CoA, NADPH)

AS a comparison of the results of experiment carried out at DTNB :enzyme ratio, 9:1 for the lane F and G shows that cross-linking was inhibited by the higher DTNB :enzyme ratio. This inhibition was probably caused by the reaction of the active-site cysteine SH group to yield a doubly modified, but non cross-linked (TNB– S_c– E)(E– S_p –TNB) enzyme dimer. Lane A is representing the Bio-Gel protein standards and molecular weights (in descending order), myosin,(200 kDa)., β-galactosidase,(116 kDa)., phosphorylase a, (97 kDa)., bovine serum albumin,(66 kDa)., ovalbumin,(45 kDa).

V.3.5 Reactivation of DTNB-inactivated FAS by treatment with DTT

Reversal of FAS activity was found to be dependent on the time of incubation of the enzyme with DTNB. As shown in (Fig. V.5), the time course of reactivation exhibited biphasic behavior. The enzyme incubated for 45 s with DTNB containing little crosslinking regained activity rapidly (96%) in 0.5 min, followed by a slower rate and complete reactivation in 2 min. Increasing the incubation time up to 180 s resulted in decrease in the rapid phase and corresponding increases in the slow phase. This biphasic behavior is consistent with the Scheme I,

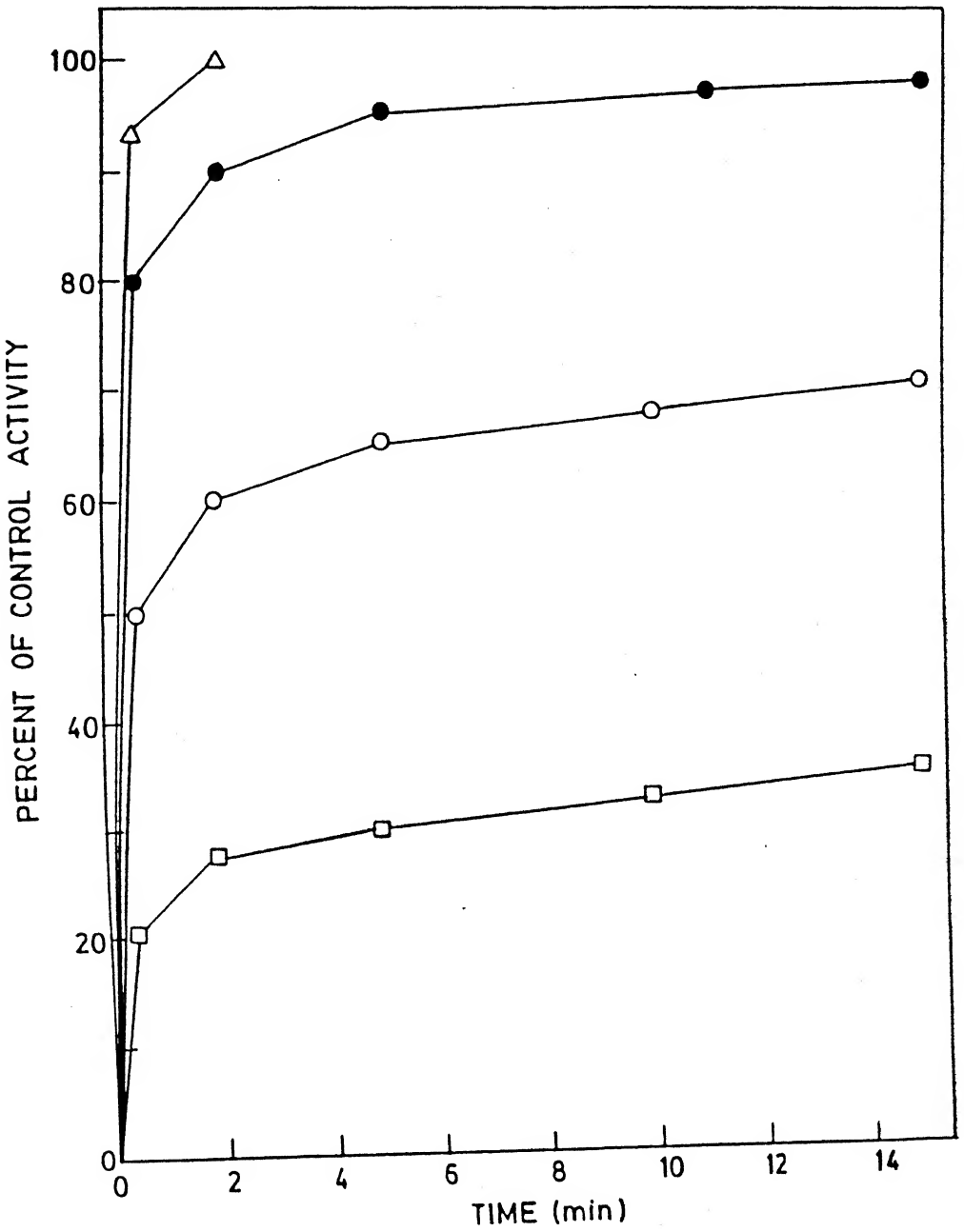
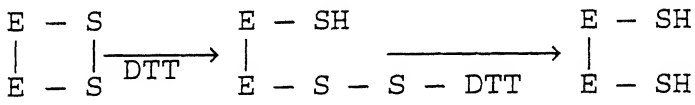


Figure V.5: *Recativation of DTNB-modified enzyme by DTT.* The enzyme (2.6 μM) was incubated with 4 μM of DTNB in 6 mM EDTA, 1% (v/v) glycerol, pH 7.0 and 25°C. FAS activity was lost in 60 sec. DTT (40 mM) was added at the following time intervals and activity recovery was determined by assays at 60 s (Δ), 90 s (●), 120 s (o) and 180 s (□).

in which the rapid phase corresponds to a single step for the removal of the TNB substituent from the inactivated enzyme (enzyme II), whereas the slower phase corresponds to a two step reaction sequence for reduction of the protein-protein disulfide (enzymeIII) such as shown in Scheme II as proposed by Hsu and Wang [27] for chicken liver FAS.



Scheme-II

V.3.6 Determination of number of SH groups involved in inactivation of FAS enzyme by DTNB

Stoichiometry of DTNB reaction with FAS was determined by measuring the residual activity at different concentrations of DTNB after the completion of reaction for 10 min. The percentage of residual activity versus DTNB concentration per enzyme molecule was plotted (Fig V.6). The plot was initially linear, but deviated from linearity at higher levels of DTNB due to the reaction of additional SH groups. Extrapolation of the plot yielded a stoichiometry of 2.0 sulfhydryl groups/dimer. The rapidly reacting essential 2.0 SH groups per

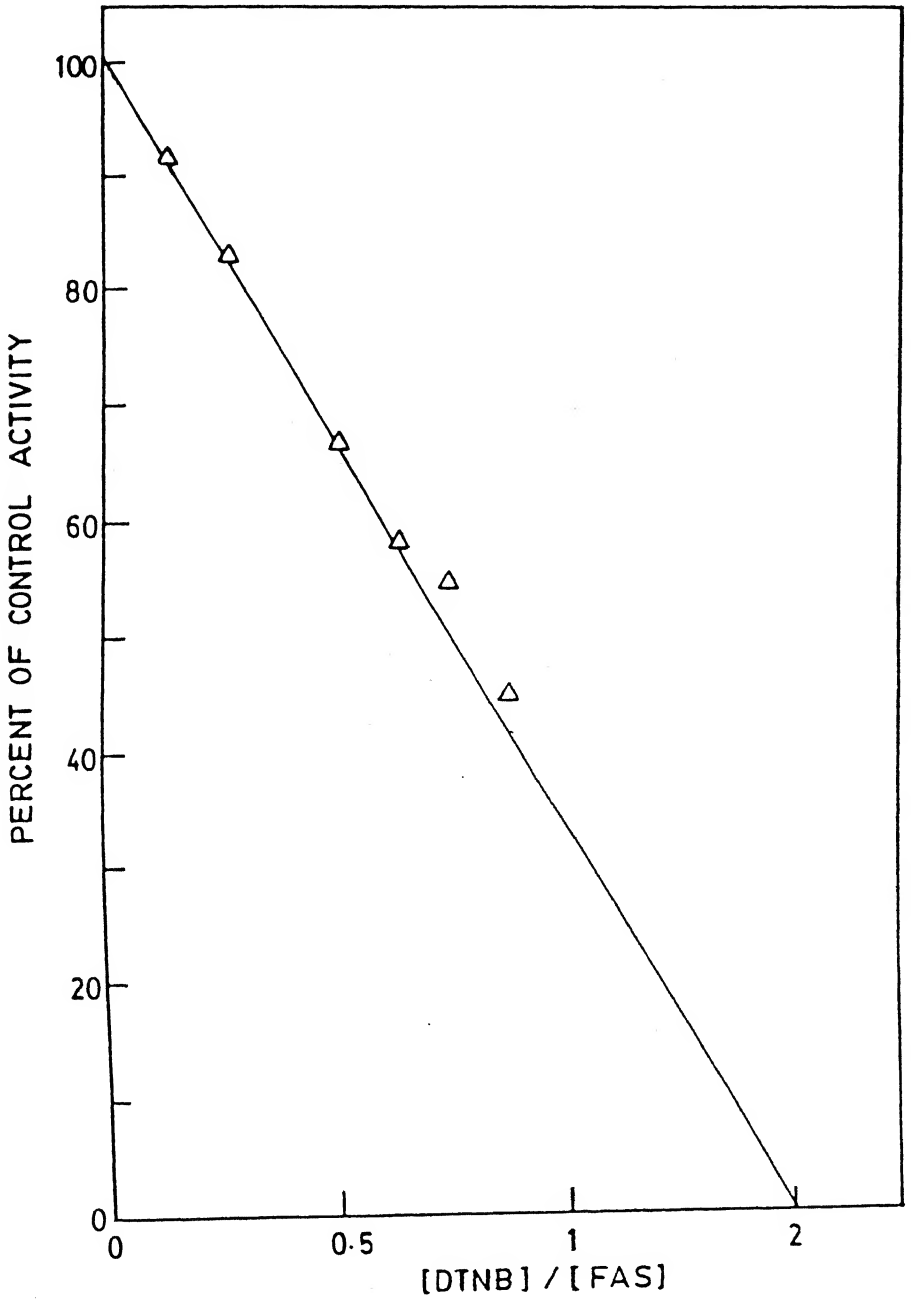


Figure V.6: *Stoichiometry of inactivation of FAS by DTNB* inactivation of FAS by DTNB. The enzyme (2.6 μM) was incubated with (1-3 μM) of DTNB in 0.2 M potassium phosphate buffer containing 1 mm EDTA and 1 mM DTT at pH 7.0 and 25°C. The residual activity was estimated after completion of the reaction in 10 min.

enzyme molecule is in agreement with the value of two phosphopantetheine SH groups reported earlier for pigeon liver FAS [28].

V.3.7 Effect of acyl-CoA esters on the time course inactivation of the FAS by iodoacetamide

The fatty acid synthetase of animal tissues has active thiol groups that are sensitive to alkylating reagents. In the earlier studies, it was found that the treatment of the synthetase with iodoacetamide has no effect on the binding of acetyl and malonyl groups to the 4'-phosphopantetheine sulfhydryl but inhibits acetyl binding to the sulfhydryl of the cysteine residue [8]. The pigeon liver fatty acid synthetase was found to be inactivated by an alkylating reagent iodoacetamide and not by iodoacetic acid. The pigeon liver FAS was completely inactivated in the presence of 0.5 mM iodoacetamide with a rate constant of 0.06 min^{-1} and $t_{1/2}$ of 5 min (Fig V.7). The reaction followed first order kinetics till 90% inhibition of enzyme activity and is therfore consistent with the assumption that this inhibition resulted from the reaction of iodoccetamide with one catalytic site of the enzyme. Earlier radiolabelled study by Wakil and Stoops, showed that the inhibition of synthetase enzyme by iodoacetamide was due to alkylation of an active cysteine thiol and this thiol

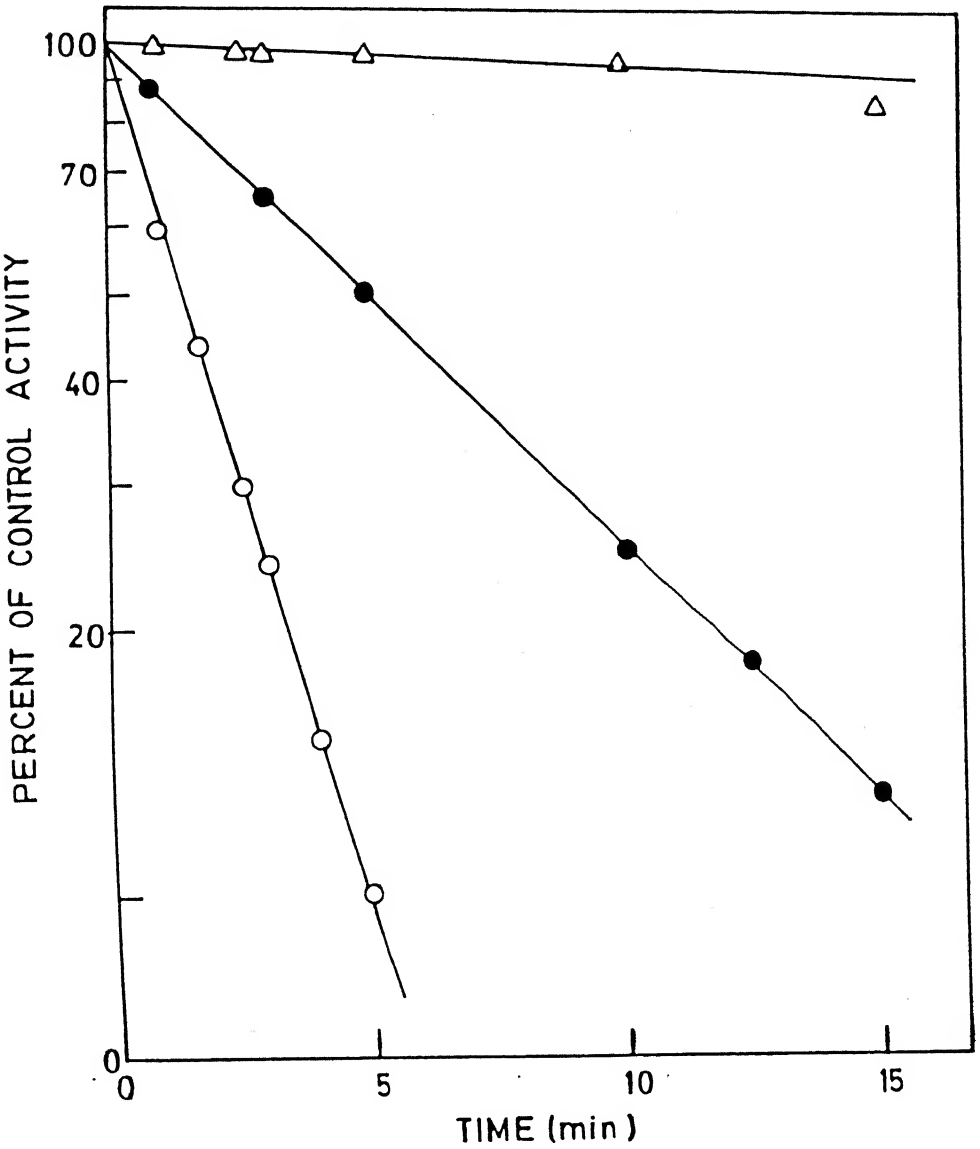


Figure V.7: *Semilogarithmic plot of the time course of the inhibition of FAS by iodoacetamide* The enzyme (1 mg/ml) was treated with 0.5 mM iodocaetamide in o.2 M potassium phosphate buffer pH 7.0, containing 1 mM EDTA in the presence of acetyl-CoA (Δ) or in the absence of acetyl-CoA (●) or malonyl-CoA (o).

was identified as the active cysteine-SH of the β-ketoacyl synthetase component [29]. Preincubation of the pigeon liver FAS enzyme with acetyl-CoA protects the enzyme from inhibition by iodoacetamide (Fig V.5), suggesting that cysteine thiol is the site of binding of the acetyl group to the β-ketoacyl synthetase. However, preincubation of the enzyme with malonyl-CoA prior to treatment with iodoacetamide did not protect the FAS against iodoacetamide inhibition, rather accelerated the inhibition with an apparent first order rate constant of 0.2 mim^{-1}, suggesting that the site of binding of malonyl group to the enzyme is not the active thiol site where the iodoacetamide is reacting. Similar results were also found with iodoacetamide inhibition of the chiken liver FAS [28].

V.3.8 Inactivation of FAS by a bifunctional reagent o-phthalaldehyde

o-phathalaldehyde, a bifunctional cross linking reagent has been used for the characterization of the nature of active site of enzymes. It forms an isoindole derivative with a characteristic absorption maxima at 337 nm by cross linking ε-amino group of lysine and sulfhydryl group of cysteine located in close proximity, (not more than 3Å apart). Pigeon liver FAS was found to be inactivated irreversibly by

stoichiometric concentrations of o-phthalaldehyde. Time course of inactivation of FAS by different concentrations of o-phthalaldehyde is shown in (Fig V.8). The rate of inactivation was dependent on the concentration of o-phthalaldehyde. Since the inhibitor concentration used was not in large excess over the concentration of enzyme, the inactivation followed a bimolecular process as found in the DTNB reaction.

V.3.9 Effect of substrates on o-phthalaldehyde inactivation of FAS

In order to locate the essential -SH and ε-NH_2 groups of cysteine and lysine, respectively, protection experiments were carried out. Acetyl-, or malonyl-CoA and NADPH protected the enzyme partially against o-phthalaldehyde inactivation as shown in (Table V.3). The extent of protection increased when a combination of acetyl-CoA or malonyl-CoA with NADPH was used. Since, the complete protection against o-phthalaldehyde inhibition was not achieved by the substrates, it was not possible to determine whether, all the residues modified are located at the substrate binding site. In the Chapter-III it has been reported that the essential lysine residue is present in the NADPH binding site of the enoyl-CoA reducatase domain of pigeon liver FAS. o-phthalaldehyde did not inhibit the

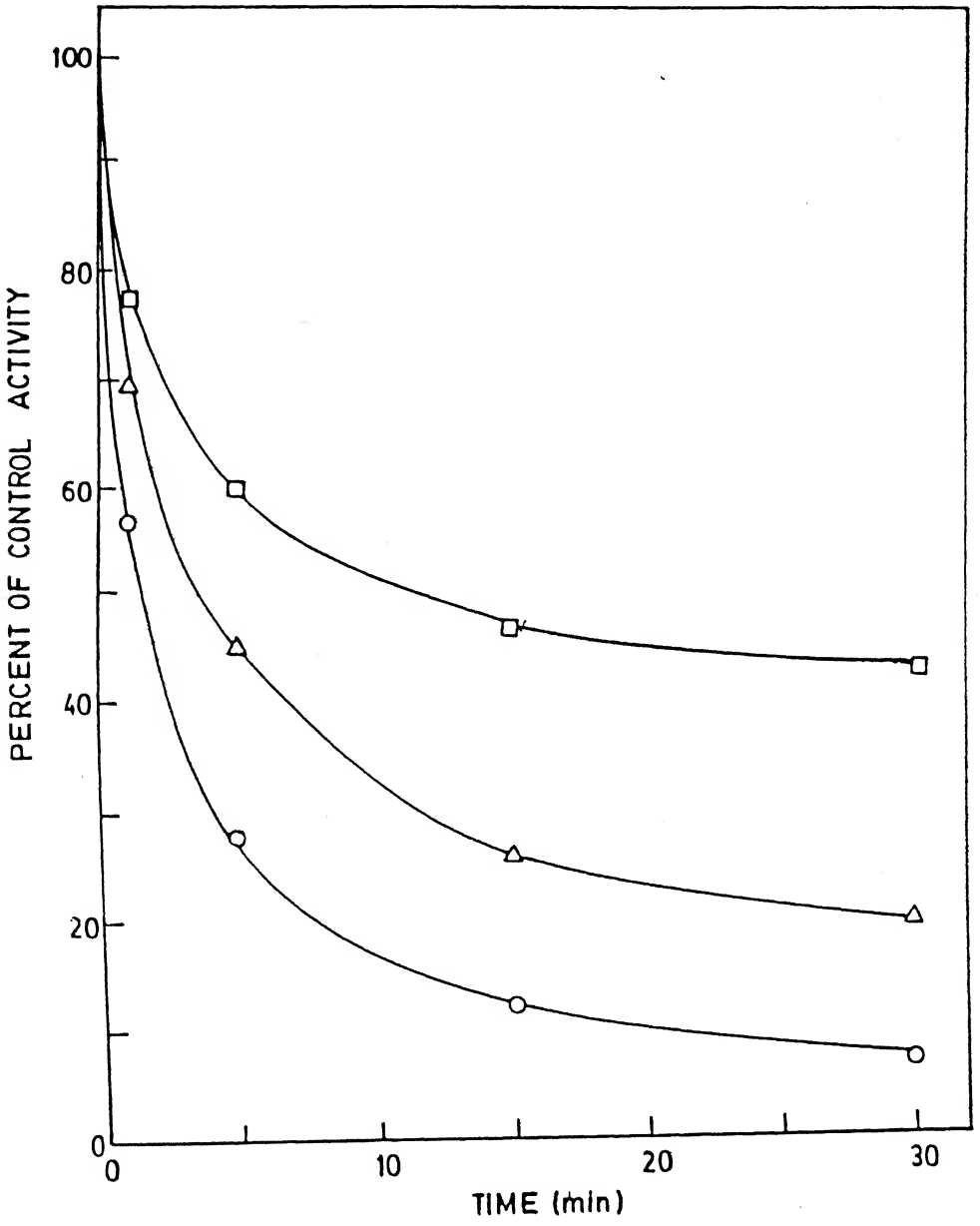


Figure V.8: *Time course inactivation of FAS by o-phthalaldehyde.* The enzyme (8 μM) was incubated in 0.2 mM potassium phosphate buffer(pH 7.0) containing 1 mM EDTA with 8 (□), 16 (Δ), and 24 (o) μM of o-phthalaldehyde. Aliquots were taken at time intervals and assayed the residual activity as described in the "Experimental Procedures".

enoyl-CoA reductase activity of the FAS, which indicated that the lysine involved in o-phthalaldehyde reaction is not essential and protection afforded by NADPH may be due to the ligand-induced transition of FAS conformers affecting the reactivity of the suceptible SH group as found in the inhibition of FAS with a thiol specific reagent, DTNB.

Table V.3

Effect of substrates against FAS inactivation by o-phthalaldehyde

The enzyme (8 μM) was preincubated with different substrates at indicated concentrations in 0.2 M potassium phosphate buffer (pH 7.0) for 15 min each. This mixture was allowed to react with 16 μM of o-phthalaldehyde for 15 min. The remaining activity was measured as described in section V.2.

SUBSTRATES	PERCENT OF CONTROL ACTIVITY
None	15
Acetyl-CoA (200 μM)	70
Malonyl-CoA (200 μM)	68
NADPH (1 mM)	55
Acetyl-CoA (200 μM) + NADPH (1 mM)	80
Malonyl-CoA (200 μM) + NADPH (1 mm)	85

V.3.10 Characterisation of isoindole formation due to fatty acid synthetase and OPA reaction

The fluorescence emission spectrum of FAS-o-phthalaldehyde adduct showed a characteristic flurorescence maxima at 412 nm when excited at 337 nm and flurescence excitation spectrum (λ_{em}=412 nm) showed a maxima at 337 nm (Fig V.9). These results were consistent with the isoindole derivative formation which involves the participation of proximal thiol and ε-amino groups of cysteine and lysine residues, respectively [15,18]. The fluorescence intensity did not change under the high salt and low salt conditions and even when recorded after 24 h. This showed that the adduct was stable and o-phthalaldehyde reaction with enzyme forming isoindole derivative does not depend on the salt concentration, unlike the DTNB reaction with FAS. The molar transition energy (E_T) was calculated by the following equation [15]

$$E_T = 2.985\lambda_{em} - 1087.28$$

E_T for this system was found to be 142.5 kJ/mole. The value of the molar transition energy of FAS and o-phthalaldehyde adduct is close to that of synthetic isoindole in dioxane [15,18] indicating that the microenvironment around the cysteine and

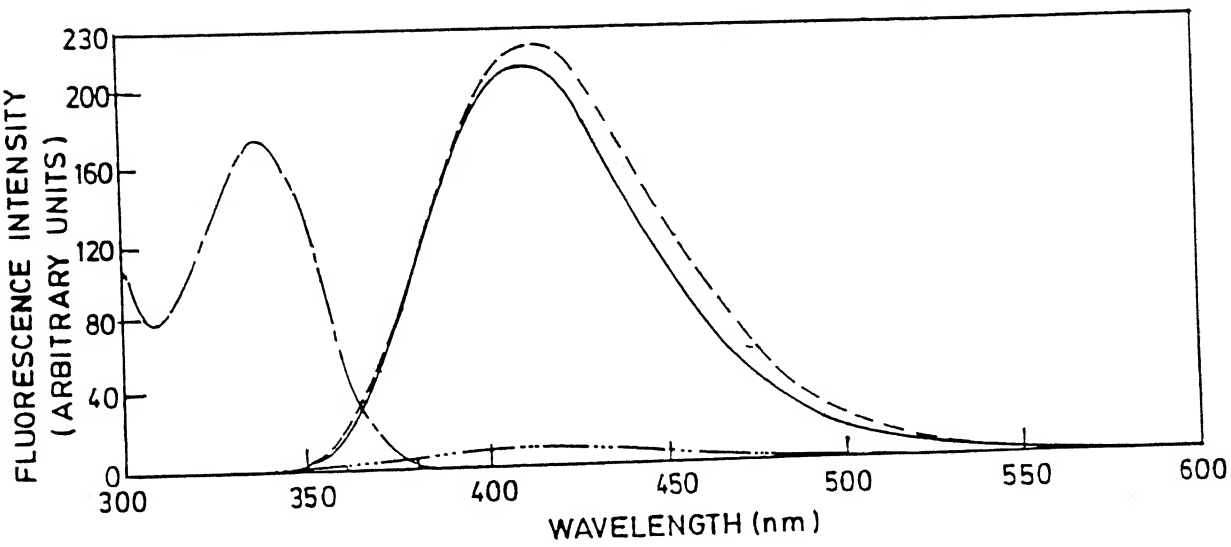


Figure V.9: *Fluorescence emission spectrum on excitation at 337 nm (———) of OPA modified fatty acid synthetase under the high salt condition and the spectrum under the low salt condition (-----). Fluorescence excitation spectrum* (λ_{em} =412 nm) (—·—) *of OPA modified enzyme and the emission spectrum of pure enzyme(—···—) on excitation at 337 nm.* FAS (4 mg/ml) was incubated with (16 μM) OPAunder the reaction conditions for 30 min. For details, see "Experimental Procedure".

lysine residues involved in isoindole formation is relatively hydrophobic in nature.

V.3.11 Spectral analysis of o-phthalaldehyde-modified FAS in presence of different inhibitors and substrates

In the protection experiments it was found that FAS is only partially protected by acetyl-.or malonyl-CoA against the o-phthalaldehyde inactivation. Protection by acetyl-CoA, or malonyl-CoA was further corroborated by the decrease in the fluorescence intensity (Fig V.10). The FAS was inactivated by DTNB and TNBS by modifying phosphopantetheine-SH groups and lysine residue. In order to identify which -SH and NH_2 groups are taking part in the reaction with o-phthalaldehyde, the FAS enzyme was treated with DTNB, iodoacetamide and TNBS in three different samples followed by o-phthalaldehyde and fluorescence spectra were recorded as shown in (Fig V.11). It was interesting to note that FAS preincubated with DTNB in stoichiometric concentration for 30 s, a period where the enzyme was in uncrosslinked state (Scheme-I) having cysteine-SH group free to react did not show any isoindole formation with o-phthalaldehyde. This indicated that o-phthalaldehyde binds at the DTNB binding site i.e to the phosphopantetheine-SH group and, not the cysteine-SH. Similar result was observed when FAS

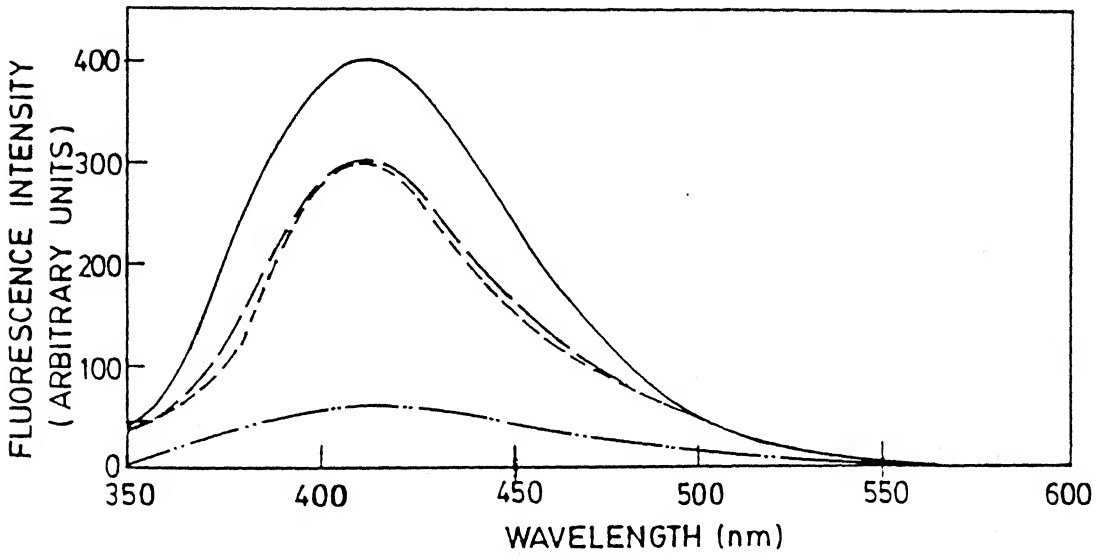


Figure V.10: *Effect of substrates on fluorescence emission spectra of OPA modified fatty acid synthetase on excitation at 337 nm using acetyl-CoA (— — —) and malonyl-CoA (----) and the emission spectrum of pure enzyme(—···—) on excitation at 337 nm. Fluorescence emission spectrum on excitation at 337 nm of OPA modified fatty acid synthetase is (———).* FAS (4 mg/ml) was incubated with (16 μM) OPA under the reaction conditions for 30 min. For details, see "Experimental Procedure".

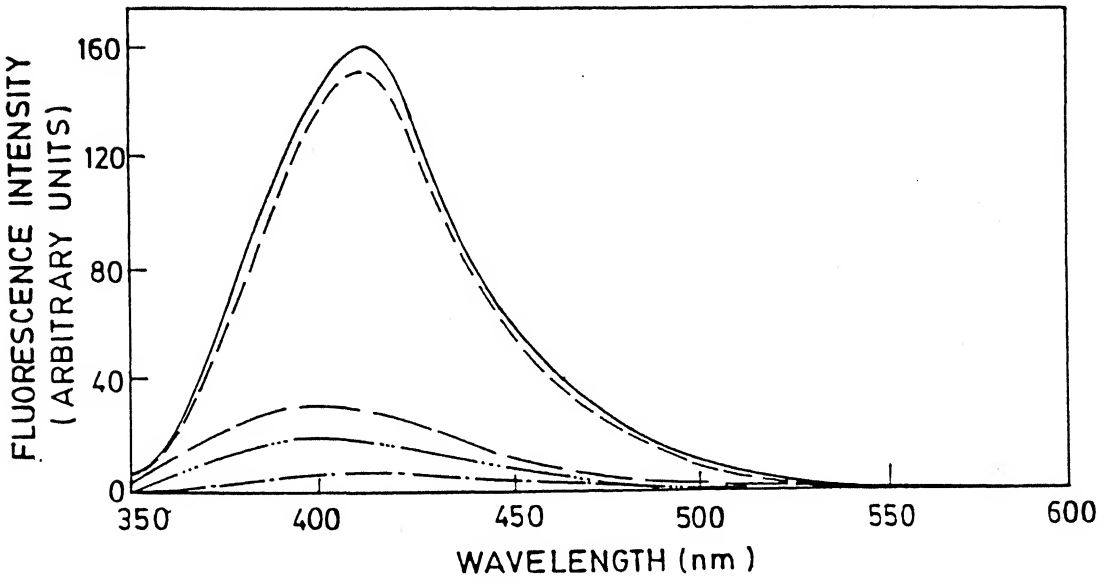


Figure V.11: *Effect of iodoacetamide (----), DTNB (——···——) and TNBS (—— ——) pretreatment of FAS on fluorescence emission spectra of FAS-o-phthalaldehyde adduct. Fluorescence emission spectrum on excitation at 337 nm (———) of OPA modified fatty acid synthetase and the emission spectrum of pure enzyme(—·—) on excitation at 337 nm.* The enzyne (1.17 mg/ml) was incubated with iodoacetamide (0.5 mM), DTNB (4μM) and TNBS (1mM) separately for 30 min, 30 sec and 30 min respectively prior to incubation with 24 μM o-phthalaldehyde for 30 min. For details, see "Experimental Procedures".

was reacted with DTNB for 5 min, a condition where pantetheine and cysteine sulfhydryl groups of FAS are cross linked. To examine the possibility of binding of o-phthalaldehyde to the cysteine-SH site, the enzyme was treated with iodoacetamide before the treatment with o-phthalaldehyde. The fluorescence intensity was almost similar to that obtained with the enzyme when treated with o-phthalaldehyde only. Experiments with prior incubation of FAS with TNBS followed by o-phthalaldehyde incubation showed significant loss of fluorescence intensity, indicating that both the inhibitors are binding to the same lysine residue. As o-phthalaldehyde does not inhibit enoyl-CoA reductase activity of the FAS enzyme, it can be concluded that the non-essential lysine residues which were modified by TNBS are taking part in o-phthalaldehyde reaction. It is reasonable also to consider that the essential lysine residue cannot react with o-phthalaldehyde because enoyl-CoA reductase and phosphopantetheine-SH group are present in different domain of the synthetase molecule and the distance between -SH group and essential $-NH_2$ group must be more than 3Å.

V.3.12 Stoichiometry of the reaction of FAS with o-phthalaldehyde

The stoichiometry of inhibition by o-phthalaldehyde was

determined by absorbance increase at 337 nm. The percentage of residual enzyme activity was plotted as a function of amount of o-phthalaldehyde incorporated per mol of the enzyme (Fig V.12). Approximately 2 moles of isoindole derivatives were formed per mol of enzyme when the data were extrapolated to zero enzyme activity.

V.3.13 Double inhibition studies

In order to determine whether the inactivation of FAS results from the binding of DTNB and o-phthalaldehyde to the same -SH group of the enzyme, double inhibition studies were carried out. As shown in (Fig V.13) the FAS activity inhibited by DTNB was completely reversed by DTT (bar 1,2). However, DTT had no effect on o-phthalaldehyde inactivated enzyme (bar 3,4). When o-phthalaldehyde was first incubated with FAS for 1 min followed by DTNB for 30 s partial reversal of enzyme activity was obtained (bar 5,6). But, the reversed order of addition of inhibitors led to the complete restoration of catalytic activity (bar 7,8). The results indicated that when FAS treated with DTNB first, it blocked the reaction of o-phthalaldehyde with the essential thiol group i.e phosphopantetheine-SH, by binding to it and when DTT was added the loss of activity was completely reversed. Thus both the reagents bind to the same

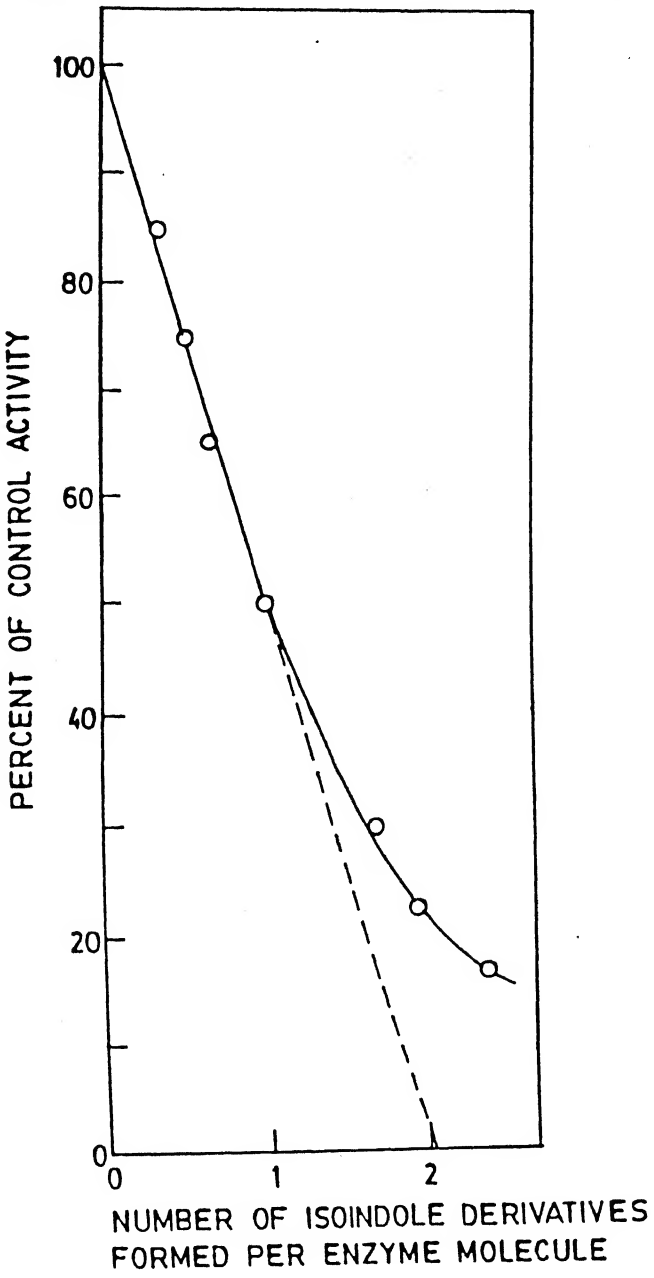


Figure V.12: *Stoichiometry of inactivation of FAS by o-phthalaldehyde.* The enzyme (8μM) was incubated with 16μM o-phthalaldehyde in 0.2 M potassium phosphate buffer (pH 7.0). The number of isoindole derivative formed at different time intervals during enzyme modification process was determined by taking the absorbance at 337 nm. In parallel experiment the aliquots were withdrawn from the incubation mixture at various time intervals and the residual activity was determined as described in "Experimental Procedure".

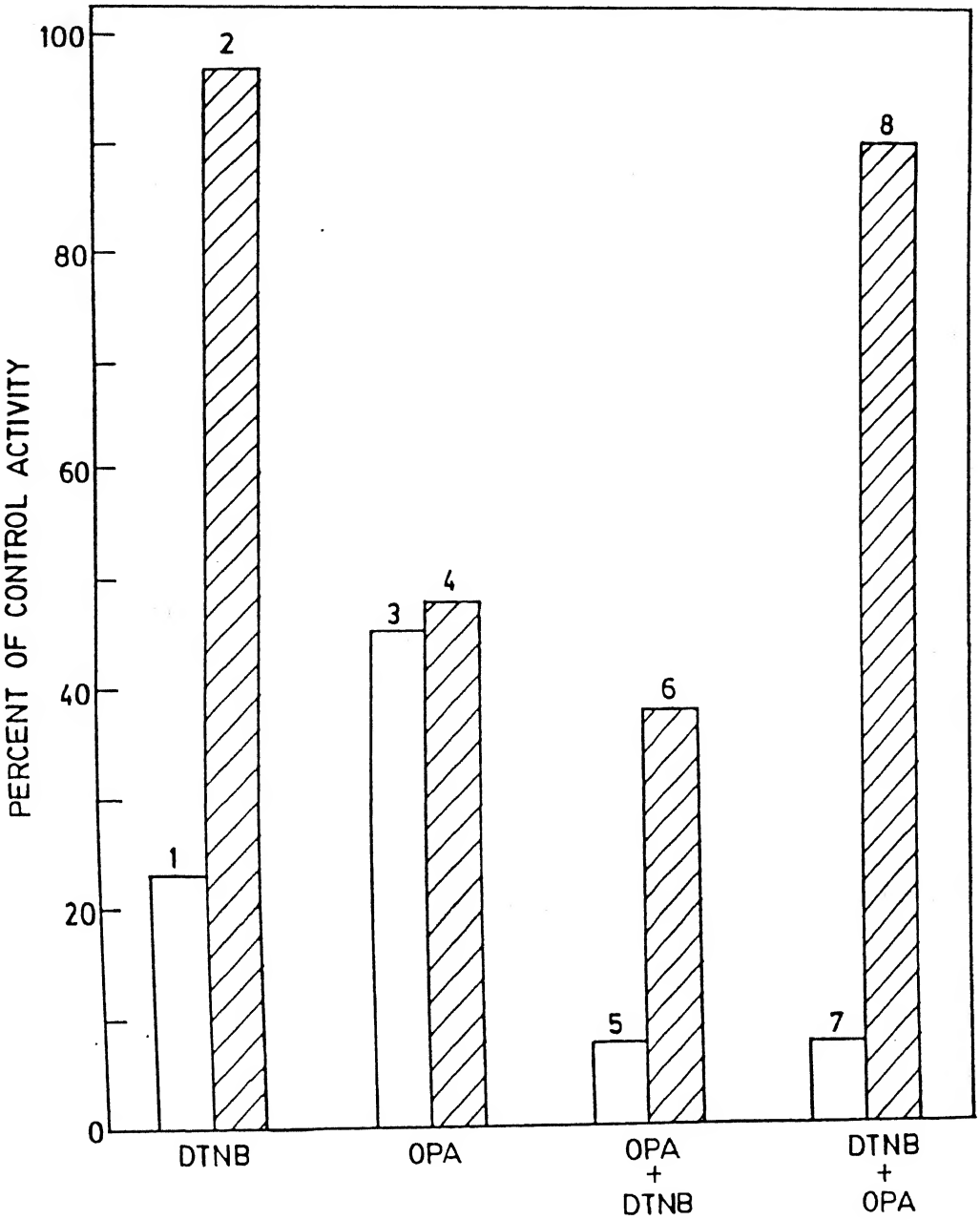


Figure V.13: *Effect of dithiothreitol on the inactivation of FAS by DTNB and o-phthalaldehyde.* The enzyme (2·6 μM) was incubated with DTNB (4 μM) for 30 s (bar 1,2), followed by 24 μM OPA for 1 min (bar 3,4), or 24 μM OPA for 1 min followed by 4 μM DTNB for 30 S (5,6) or 4 μM DTNB for 30 s followed by 24 μM OPA (bar 7,8). At the end of indicated treatments the residual activity was determined. The remaining incubation mixtures was freed from excess reagent by column -centrifugation and allowed to react with DTT (40 mM) for 5 min. The time course of recovery of activity was then followed.

essential thiol group present in enzyme, signifying the critical involvement of this phosphopantetheine thiol in the catalytic activity.

V.4 Summary

Chemical modification methods can be employed successfully to identify amino acid residues that are important for enzyme activity provided that the following two criteria are fulfilled: First, chemical modification of these amino acid residues must result in loss of activity. Second, this inactivation must be prevented by substrate or their analogs. Fatty acid synthetase was inactivated by a thiol specific reagent, DTNB and by a bifunctional reagent o-phthalaldehyde. The inactivation by both the inhibitors followed a bimolecular reaction involving phosphopantetheine-SH group as confirmed by the protection, fluorometric and double inhibition studies. DTNB inactivation was highly dependent on salt concentration due to its conformational changes. In the low salt conformer phosphopantetheine -SH and cysteine -SH groups are juxtaposed, and the DTNB reaction and dimerization are both facilitated. Transition to the high salt conformer is accompanied by reduction of reactivity with DTNB and reduced demerization which probably is due to spatial separation of the -SH groups.

However, the o-phthalaldehyde reaction does not depend on salt concentration.

Inactivation of FAS by DTNB and o-phthalaldehyde was prevented in the presence of acetyl-CoA, malonyl-CoA and NADPH. While the substrates (acetyl-CoA , malonyl-CoA) protect the reaction of phosphopantetheine -SH group with modifying agents, NADPH protection was probably due to ligand induced transition of FAS conformers affecting the reactivity of SH group. Inactivation of FAS by DTNB could be reversed by DTT. However, the rate of reactivation was dependent on the initial incubation period of the enzyme with DTNB. The inactivation by o-phthalaldehyde was not reversed by DTT.

FAS was also inactivated by alkylating reagent, iodoacetamide. Earlier studies indicated that cysteine-SH group of FAS can only bind acetyl group but not malonyl group during fatty acid synthesis. The protection against the inactivation of iodoacetamide by acetyl-CoA but not by malonyl-CoA confirmed the fact that this inhibitor binds specifically to cysteine -SH group.

Fluorescence spectral studies showed that pretreatment of FAS with DTNB or TNBS prior to the addition of o-phthalaldehyde prevented the isoindole formation. On the other hand pretreatment of FAS with iodoacetamide did not affect the

isoindole formation as there was no significant diffrence in the intensity of fluorescence spectra of FAS-o-phthalaldehyde adduct in the presence or the absence of iodoacetamide. These results clearly show that pantetheine -SH group is reacting with o-phthalaldehyde.

FAS reaction with o-phthalaldehyde leads to the formation of isoindole derivative by the reaction of proximal -SH and $-NH_2$ group. Participation of essential pantetheine -SH group in isoindole formation was ascertained based on protection and fluorescence spectral studies. Interestingly o-phthalaldehyde did not affect the enoyl-CoA reductase activity of FAS complex, which has an essential $-NH_2$ group. Thus amino group participating in the isoindole formation during the inactivation of FAS by o-phthalaldehyde is not essential. It was observed that TNBS, a $-NH_2$ group specific reagent modifies 2 lysine residues per subunit of FAS and only one of these lysine is essential (chapter III). Since TNBS prevented the isoindole formation it is possible that the other TNBS reactive $-NH_2$ group is reacting with o-phthalaldehyde along with pantetheine -SH group to form isoindole.

Stoichiometric studies with o-phthalaldehyde have showed that two moles of isoindole derivative were formed upon complete inactivation by o-phthalaldehyde. similar

stoichiometry was also found with DTNB reaction.

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